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PURIFICATION AND CHARACTERIZATION OF THE VIRULENT FACTOR LIPASE EXTRACTED FROM *CANDIDA CATENULATA*

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Abstract

The molecular identification using 26S ribosomal RNA indicated that the *Candida* species in this study was *Candida catenulata* strain QD16.1. Treatment of *C. catenulata* with different antifungals revealed that fluconazole (F) was the most inducer to the virulence factor lipase followed by itraconazole (I), then miconazole (M), while combination of (F) and (I) synergistically inhibited lipase induction, so it reduce *Candida* virulence. Purification of lipase extracted from *C. catenulata* growth medium was performed using ammonium sulfate [$(NH_4)_2SO_4$] precipitation and Sephadex G100 gel chromatography which resulted in one peak of purified enzyme. The pure enzyme detected that the optimum pH was 5.5, temperature was 37°C. The value of K_m was 0.271 mM and V_{max} was 14.94 U/ml. The metal ions Na^+, K^+, Ba^+ and Fe^{+2} inhibited lipase activities, while Hg^{+2} strongly reduced it. However, Zn^{+2} exerted an enhancement to lipase activity. EDTA and β-mercaptoethanol inhibited lipase activity indicating that it is a metalloenzyme. The detergents Tween 80 and SDS enhanced its activity, while Tween 20 and Triton x-100 inhibited it.

Key words: *C. catenulata*, lipase, antifungals.

Introduction

Formerly, Candida catenulate (Diutina catenulata) is considered as an ascomycetous yeast obtained from humans, animals, and sources of the environment. The organism is a contaminant in dairy foods and has been correlated with both human and animal superficial and invasive infections (O'Brien *et al.,* 2018). It was isolated from soil in Dublin and Ireland by Sylvester *et al.,* (2015). Candida catenulata is commonly associated with dairy products like milk (Delavenne *et al.,* 2011) and cheese (Facchin *et al.,* 2013; Gkatzionis *et al.,* 2014). Candida also was isolated from the microbiota in the oral cavity (Santin *et al.,* 2013), from the gastrointestinal tract and poultry feces (Subramanya *et al.,* 2017), from wild birds (Mendes *et al.,* 2014; Subramanya *et al.,* 2017; Brilhante *et al.,* 2017) and piglets (Urubschurov *et al.,* 2008). Its environmental niche is not known, although it was identified in rural dust (Janke *et al.,* 2013) and the Tagus river estuary (de Almeida, 2005).

C. Catenulata was associated with human diseases including some patients with cancer (Radosavljevic *et al.,* 1999) and vulvovaginal infections (Sarbu *et al.,* 2013). *C. catenulata* could be used in bioremediation because of their hydrocarbon degradation capacity (Joo *et al.,* 2008).

Candida species is responsible for 50-80% of all instances of fungal endocarditis relative to *Aspergillus* species, which accounts for 25% of fungal endocarditis in prosthetic valve patients (Tattevin *et al.,* 2014).

Candida catenulata, known formerly as *C. ravautii*, *C. brumptii*, is a yeast usually present in dairy goods (Diddens and Lodder, 1942; Roostita and Fleet, 1996).

In recent years, extracellular enzymes of microbial pathogens have gained considerable interest for their potential function in pathogenesis and as potential targets for the production of synthetic inhibitors to treat infections. For promoting the host tissues invasions, those pathogens secrete lytic enzymes (hemolysins, proteinases, lipases, phospholipases, …) to shatter, alter, or harm the integrity of the host membranes, causing the malfunction, or rupture of the host cells (Trofa *et al.,* 2008; Sanita *et al.,*

2014). However, lipases are available from many sources, including microbes such as bacteria, fungi with yeast being the most suitable sources for lipase production. These microorganisms can produce lipases of high quality in lower cost and shorter time periods (Trichel *et al.,* 2010). Different plants, animals, and micro-organisms produce them. Microbial, bacterial and fungal lipases are also widely used in biotechnological applications (Ülker and Karaoðlu, 2012). *Bacillus*, *Arthrobacter* and *Pseudomonas* are the most common genera of bacteria used for lipase applications. Higher levels of extracellular lipases may be also produced by filamentous fungi (Saxena *et al.,* 1999), and the most frequently utilized fungal genera in applications are *Mucor*, *Penicillium*, *Aspergillus*, *Geotrichum* and *Rhizopus* (Ghosh *et al.,* 1996). *Penicillium cyclopium*, *P. brevicompactum*, *P. chrysogenum*, *P.crustosum*, *P. roqueforti* and *P. verrucosum* are the most widely used members of the genus *Penicillium* (Li and Zong, 2010). Lipases were also commonly used in the detergent, pharmaceutical, food and dairy sectors, in particular during cheese ripening (Gupta *et al.,* 2004b; Hasan *et al.,* 2006). Lipases are the third main category of enzymes after proteases and carbohydrases depending on the overall selling market (Liu *et al.,* 2008).

The optimal growth conditions should be considered for achieving maximum lipase yields (Linefield *et al.,* 1990). The development of lipase depends on a number of factors including sources of carbon, nitrogen, pH, temperature, aeration and inoculum size (Kim *et al.,* 1996; Gupta *et al.,* 2004a).

Lipases are another significant category of enzymes with substantial physiological and industrial importance. Lipases primarily catalyze the hydrolysis of fats and oils into glycerol and fatty acids (Mohamed *et al.,* 2011). Lipases (triacylglycerol acylhydrolases, E.C. 3.1.1.3) are one of the main biotechnology groups capable of catalyzing the hydrolysis of the ester bond at the lipid and water interfaces (Sharma *et al.,* 2001). Due to their commercial importance, only microbial lipases are of great interest among different sources of lipases (Shah and Bhatt, 2011). It is necessary to evaluate the lipase activity during the yield by selecting the best condition for production. Though several methods for evaluating lipase activity have been established, only two methods are widely used in the literature. The first is the titrimetric technique which uses olive oil as substrate. This system relies on the determination of the liberated fatty acids by titration against potassium hydroxide (Beisson *et al.,* 2000). The second approach is the colorimetric test using p-nitrophenyl palmitate as a substrate and evaluate the activity of lipase by calculating the amount of yellow chromogen (p-nitrophenol) resulting from substrate hydrolysis (Hasan *et al.,* 2009). Due to their simplicity, speed, the colorimetric methods are generally more desirable where only small volume of the sample is required for analysis.

The objective of this study was to extract, purify and characterize the secreted lipases by *C. catenulata*. The effect of antifungal application on the secretion of lipases was also investigated.

Materials and methods

Test organism

Candida catenulata was kindly provided by Micro Analytical Center, Cairo University.

Culture and maintainance

C. catenulata was cultured in medium composed of (%): peptone (1), glucose (2) and agar (2) and incubated at 37^0C for 48h. It was subcultured according to Bramono *et al.,* (2006) and maintained in refrigerator until use.

Lipase extraction

For lipase induction *C. catenulata* was grown for 3 days in a liquid medium containing 0.7% yeast extract (modified from yeast nitrogen base) and 2.5% tween 80 (Tsuboi *et al.,* 1996). Fifty milliliters of the culture medium contained in 200 ml flask were kept at 37 °C for 20 minutes shaking at 150 rpm and then filtered. The yeast cells were dried and weighted, while the filtrate was used for lipase assay.

Culture filtrate (0.3 ml) was added to 1.0 ml of the substrate 1.12 mM of sorbitane monopalmitate (modified from alpha-naphthylpalmitate), 14 mM of citric acid and 74 mM of Tris buffer (PH 5.5) [for 1 hr at 37°C. The reaction was stopped by adding 0.5 ml of 0.5 N NaOH solution. Sorbitol released was reacted with 0.3 ml fast violet B (1 mg/10 ml H_2O), and the optical density was measured at 520 nm on (JENWAY 6300 Spectrophotometer). Reaction mixture without culture filtrate was used as a blank. The enzyme activity was expressed in the international unit; one activity unit corresponds to the amount of enzyme that catalyzes the hydrolysis of 1µmol of substrate (sorbitane monopalmitate in the present study) per minute (Tsuboi *et al.*, 1996).

Determination of protein

The concentration of proteins was determined by the method described by Lowry *et al.* (1951).

Topic and Systemic antifungals

Micoban (Amriya Pharm. Ind.), Flucoral (SEDICO) and Itracon (Global Napi Pharmaceuticals) were used

as antifungal agents. Micoban (20 mg/g miconazole nitrate) was applied as topic antifungal, while, Flucoral (150 mg fluconazole) and Itracon (100 mg itraconazole) were used as systemic antifungals. First, lipase was assayed in culture filtrate without treatment with antifungals. Then, lipase was assayed in culture filtrate after treatment with topic and systemic antifungals, singly and in combination.

Purification and characterization of lipase from *Candida catenulata*

The crude culture filtrate of *Candida catenulata* was collected by filtration aseptically through filter paper. After that lipase activity and protein content were determined.

Ammonium sulfate precipitation and Sephadex G-100 column chromatography

Partial lipase purification was performed using different concentrations of $(NH_4)_2SO_4$ (0-100%) with continuous stirring at 4°C. The mixture was cooled centrifuged for 7 min at 6000 rpm, and the precipitated protein was dialyzed for 24 hours at 4°C in a dialysis bag against 0.1 M of tris buffer (pH 5.5). The protein content and lipase activity were estimated. The concentrated enzyme was then loaded on to a Sephadex G-100 column and balanced with a 0.1 M tris buffer (pH 5.5). The column was washed with the same buffer. The enzyme was eluted from the column at a flow rate of 1 ml / min. Fractions of enzymes (5 ml each) were obtained, and the protein content was determined and lipase assay was measured at 520 nm spectrophotometer.

Characterization of purified lipase

Effect of pH on pure lipase activity was studied using different pHs (2, 4, 4.5, 5, 5.5, 6, 6.5 and 7) adjusted by different buffers. Effect of temperature on lipase activity was investigated by incubation of the reaction mixture at different temperatures (25, 30, 35, 37, 40 and 45 °C).

Also, Effect of substrate concentration on lipase activity was studied using five different concentrations of sorbitane monopalmitate (0.56, 1.12, 2.24, 4.48 and 8.96 mM). The K_m and V_{max} were then calculated.

The effect of different metal ions and inhibitors on lipase activity was performed using (10 mM) of Nacl, KCL, $BaCL_2$, $ZnSO_4.7H_2O$, $HgCl_2$, $FeCl_3$, EDTA and â-mercaptoethanol) in 1 ml of 74 mM tris buffer (pH 5.5) at 37°C.

In addition, the effect of detergent and organic solvents on lipase activity was determined using different detergents (1% v/v) Triton x-100, Tween 20, Tween 80 and SDS and different organic solvents (acetone, methanol, isopropanol and butanol). In all experiments, the lipase activity and specific activity were determined after incubation of the reaction mixture for 1 hr at 37 °C.

Statistical analysis

Results were shown as mean ± standard deviations (mean±SD) of triplicate samples. Data were analyzed by one-way analysis of variance (ANOVAs) using SPSS statistical tools. With Duncan's test at $P \leq 0.05$, the differences between mean values were also evaluated.

Results and Discussion

Molecular identification was done for *Candida catenulata* based on 26S ribosomal RNA sequence. The constructed phylogenetic tree was shown in Fig. 1. It was found that it was *Candida catenulata* strain QD16.1.

Genus *Candida* has been identified based on 26S ribosomal RNA partial sequence and has shown to be 99.55% identical of 100% query to *Candida catenulata* strain QD16.1 with accession number: EU585766.1 in the NCBI gene bank, table 1.

Candida catenulata was described by Diddens and Lodder, (1942) to accommodate a clinical isolate originally identified as *Monilia rugosa* (nom.nud.). The conspecificity of *Candida catenulata* and *Candida brumptii* and *Candida ravautii* was established by DNA re-association experiments (Meyer *et al.*, 1978). The species cannot be assigned with confidence to any particular phylogenetic group on the basis of D1/D2 LSU

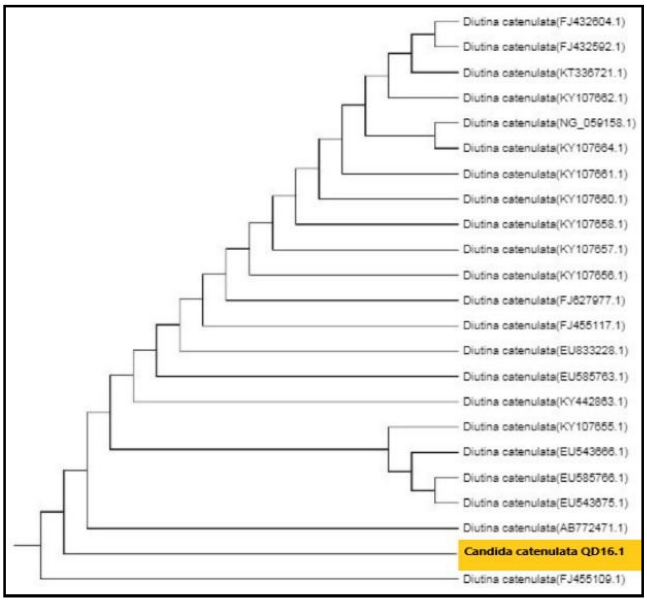

Fig. 1: phylogenetic tree showing genetic relationship between *Candida* (*Diutina*) *catenulata* strain QD16.1 (highlighted) and other reference *C. catenulate* in NCBI gene bank.

Table 1: Genetic identification of the genus *Candida* with the percentage of similarities to the resulted strains in NCBI gene bank.

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Candida catenulata strain QD16.1 26S ribosomal RNA gene, partial sequence	811	811	100%	0.0	99.55%	EU585766.1
Candida catenulata strain QD16.2 26S ribosomal RNA gene, partial sequence	811	811	100%	0.0	99.55%	EU543675.1
Candida catenulata strain 5.3-4 26S ribosomal RNA gene, partial sequence	809	809	99%	0.0	99.55%	FJ455109.1
Candida catenulata 26S ribosomal RNA gene, partial sequence	809	809	100%	0.0	99.55%	EU543666.1
Diutina catenulata CBS 565 28S rRNA gene, partial sequence; from TYPE material	804	804	100%	0.0	99.33%	NG_059158.1
Diutina catenulata culture CBS:7135 large subunit ribosomal RNA gene, partial sequence	804	804	100%	0.0	99.33%	KY107664.1
Diutina catenulata culture CBS:2743 large subunit ribosomal RNA gene, partial sequence	804	804	100%	0.0	99.33%	KY107662.1
Diutina catenulata culture CBS:7230 large subunit ribosomal RNA gene, partial sequence	804	804	100%	0.0	99.33%	KY107661.1
Diutina catenulata culture CBS:565 large subunit ribosomal RNA gene, partial sequence	804	804	100%	0.0	99.33%	KY107660.1
Diutina catenulata culture CBS:6824 large subunit ribosomal RNA gene, partial sequence	804	804	100%	0.0	99.33%	KY107658.1
Diutina catenulata culture CBS:2821 large subunit ribosomal RNA gene, partial sequence	804	804	100%	0.0	99.33%	KY107657.1
Diutina catenulata culture CBS:2014 large subunit ribosomal RNA gene, partial sequence	804	804	100%	0.0	99.33%	KY107656.1
Diutina catenulata culture CBS:2015 large subunit ribosomal RNA gene, partial sequence	804	804	98%	0.0	99.77%	KY107655.1
Candida catenulata strain CBS 565 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed s	804	804	100%	0.0	99.33%	KT336721.1
Candida catenulata gene for large subunit ribosomal RNA, partial sequence, strain: DMKU-RK81	804	804	100%	0.0	99.33%	AB772471.1
Candida catenulata isolate FSMP-Y25 26S ribosomal RNA gene, partial sequence	804	804	100%	0.0	99.33%	FJ627977.1
Candida catenulata strain 3.5-2 26S ribosomal RNA gene, partial sequence	804	804	100%	0.0	99.33%	FJ455117.1
Candida catenulata strain L2.1 26S ribosomal RNA gene, partial sequence	804	804	100%	0.0	99.33%	EU833228.1
Candida catenulata strain QD9.2 26S ribosomal RNA gene, partial sequence	804	804	100%	0.0	99.33%	EU585763.1
Diutina catenulata isolate M1.1 large subunit ribosomal RNA gene, partial sequence	802	802	97%	0.0	100.00%	KY442863.1
Candida catenulata strain 11-2 26S ribosomal RNA gene, partial sequence	802	802	99%	0.0	99.32%	FJ432604.1
Candida catenulata strain 3.1-3 26S ribosomal RNA gene, partial sequence	802	802	99%	0.0	99.32%	FJ432592.1

(Kurtzman and Robnett, 1998) or SSU (Sugita and Nakase, 1999) rRNA gene sequences, although it undoubtedly belongs to the Saccharomycetales.

Assay of lipase activity *by C. catenulata* in presence of antifungals either singly or in combination

The data in table 2 revealed an increase in the secretion of the virulence factor lipase by *C. catenulata* in the presence of fluconazole and itraconazole antifungals indicating their effectiveness on increasing fungal pathogenicity. But when they were added in combination, synergistic inhibition action was exerted on lipase activity, which reduces *C. catenulata* virulence. The same reduction in pathogenicity was exhibited when miconazole was added to the growth medium of *C. catenulata*.

In this connection, fluconazole alone and in combination with clotrimazole can induce changes in the levels of expression of hydrolytic enzymes (LIP1, LIP4, SAP2 and SAP4), which are recognized as significant virulence factors for *C. tropicalis* pathogenicity as reported by Zaugg *et al.*, (2001); Silva *et al.,* (2011); Zuza-Alves *et al.*, (2017); Richardson *et al.*, (2018) and Khodavandi *et al.*, (2019). Also, Gu *et al.*, (2016) showed that fluconazole in combinations with fluoxetine made SAP1-4 gene expression down-regulation in *C. albicans* strains. Bramono *et al.*, (2006) demonstrated that *C. albicans* followed by *C. parapsilosis* and *C. tropicalis* showed higher proteinase and lipase activity levels in each nutrient restricted medium. It is interesting to mention that all azoles contain a free nitrogen atom which binds

Table 2: Assay of lipase activity, protein content and specific activity of *Candida catenulata* in presence and absence of different antifungal drugs.

Antifungal agents	Lipase activity (U/ml)	protein conc. (mg)	Specific activity (U/mg)
Control (no antifungal)	17.26 ± 1.38^{bc}	2.47 ± 0.150^{bc}	7.0
Fluconazole (F)	20.31 ± 3.86^{c}	2.24 ± 0.189^{a}	9.01
Itraconazole (I)	20.25 ± 1.82^{c}	2.79 ± 0.443^{c}	7.33
Miconazole (M)	15.22 ± 1.33^{b}	2.27 ± 0.202^{a}	6.74
F+I	10.81 ± 1.07^{a}	2.34 ± 0.088^{bc}	4.62

$P \leq 0.05$; each value represent the mean of triplicates ± SD.

to the heme group at the active site of Erg11 enzyme that is encoded by the ERG11 gene in Candida. Thus it inhibits the heme-protein, which in turn affects the fungal cytochrome P450 enzyme 14α-demethylase of lanosterol. The latter is a rate-limiting enzyme in the fungal biosynthetic pathway of ergosterol. This action damages cell divisions, destabilizes the fungal cell membrane through inhibiting several membrane-bound enzymes and the membrane lipid biosynthesis (Sheehan *et al.,* 1999; Hitchcock and Whittle, 1993) causing cell content leakage, lysis and eventual death (Ghannoum and Rice, 1999).

Purification and characterization of lipase extracted from *C. catenulata*

The crude extract of *C. catenulata* had shown lipase activity of 280 U/ml; protein of 88.6 mg and specific activity of 3.16 U/mg.

Protein precipitations using different $(NH_4)_2 SO_4$ concentrations indicating that saturations were attained at 60 % $(NH_4)_2 SO_4$. After desalting of the enzyme by dialysis overnight, lipase activity reached 231.5 U/ml with specific activity 5 U/mg. the purification fold was 1.58 with recovery of 82.6 %. The dialyzed enzyme was subjected to gel filtration chromatography using Sephadex G-100. One activity peak of pure enzyme including (7, 8, 9, 10) fractions with gained activity of 121 U/ml, specific activity of 6.36 U/mg, purification fold of 2.01 and recovery of 43.2%, was recorded.

Similarly, Tripathi *et al.*, 2014 mentioned that *Microbacterium* sp extracellular lipase was purified by precipitation of $(NH_4)_2 SO_4$ and SephadexG-100 column chromatography with a total yield of 20.8 per cent and 2.1 purification fold. In addition, Jayaprakash and Ebenezer, (2012) recorded that lipase activity (after 70 % saturation with $(NH_4)_2 SO_4$ in *Aspergillus japonicus* was approximately 367.14 U/mg. The total protein yield was 21 mg, specific activity was 480 U / mg protein with 3.44 purification fold and 4.53 % yield.

Biochemical characterization of the purified lipase from *Candida catenulata*

Effect of pH

The optimum pH for purified lipase from *Candida catenulata* is 5.5 with enzyme activity 13.25 U/ml and specific activity of 6.30 U/mg. At pH 8 and pH 2, lipase lost 46.03 % and 18.94 % of its activity, respectively Fig. 3.

Similar to the present result, the pH 5.5 was found to be optimum for fungal lipases such as *Aspergillus oryzae, Humicola lanuginosa and Neurospora* sp. TT-241 (Omar, 1987; Lin, 1996; Toida, 1995). Also, Crueger and Crueger (1993) and Falony *et al.*, (2006) reported that maximum lipase activity for some *A. niger* strains occurred at pH 6. However, optimum lipase activity was pH 7 for *Mucor hiemalis* (Hiol *et al.*, 1999), pH 8.5 for *Trichoderma harzianum* and *Aspergillus carneus* (Ulker *et al.*, 2011; Saxena *et al.*, 2003), and pH 9 for *Penicillium caseicolum* (Saxena *et al.*, 2003). It is important to know that Amino acids are amphoteric and

Table 3: Purification protocol of *Candida catenulata* lipase.

Purification step	Total activity (U/ml)	Total protein (mg)	Specific activity (U/mg)	Purification fold	Recovery (%)
Crude extract	280±2.00^c	88.6±0.404^c	3.16	1	100
$(NH_4)_2SO_4$	231.5±0.500^b	46.3±4.04^b	5	1.58	82.6
Sephadex G-100	121±1.00^a	19±1.00^a	6.36	2.01	43.2

P ≤ 0.05; each value represent the mean of triplicates ±SD

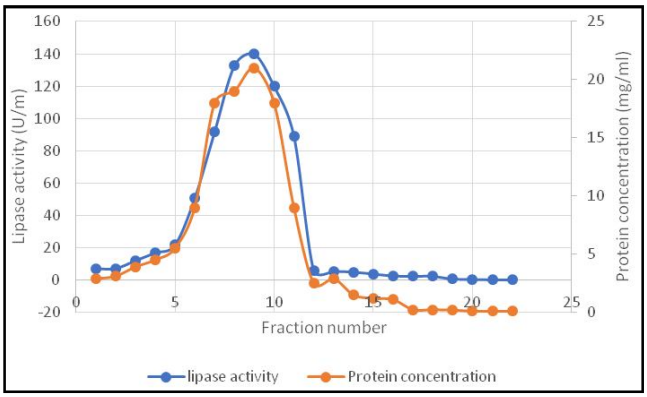

Fig. 2: Typical elution profile for the chromatography of lipase on Sephadex G-100 column.

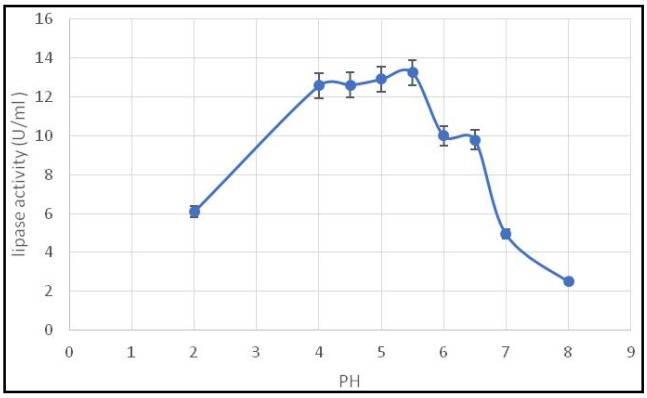

Fig. 3: Effect of pH on lipase activity purified from *C. catenulate*.

can both accept hydrogen ions and lose hydrogen ions which allow them to resist pH changes. But the tertiary structure of the lipase is sensitive to changes in pH. It has an optimum pH at which it works most efficiently due to its exact arrangement of the active site as it is partly fixed by hydrogen and ionic bonds between -NH2 and -COOH groups of the polypeptides that make it up. As the pH is considered as a factor of enzymes stability so, small changes in it, affect this bonding causing changes of shape in the active site, leading to the enzyme denaturation and it can no longer bind to its corresponding substrate (Williams, 2000).

Effect of temperature

The optimum temperature for purified lipase was found to be 37°C. Higher temperature (45°C) or lower temperature (15°C) led to decrease in enzyme activity of 5.04 % and 23.2 %, respectively. The enzyme was considered as mesophilic lipase Fig. 4.

Similar to the present results, Kamini *et al.*, (1998) characterized *A. niger* lipase extracted from gingelly oil cake and found that it has maximum enzyme activity at pH 7 and 37°C. Saxena *et al.*, (2003) noted that *Aspergillus carneus* lipase

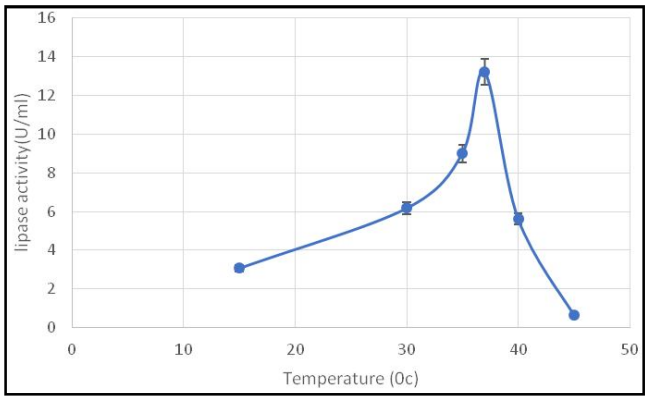

Fig. 4: Effect of temperature on purified lipase from *C. catenulata*.

exhibits optimum temperature of 37°C. Also, Das *et al.*, (2016) found *Aspergillus tamari* purified lipase exhibited maximal activity was at 37°C. However, the optimum temperature for lipase activity of *Trichoderma harzianum* (Ulker *et al.*, 2011) and *Aspergillus japonicus* (Jayaprakash and Ebenezer, 2012) was found to be 40°C and the enzyme preparation was observed to have very high activity at temperatures ranging from 30°C to 50°C. Similarly, lipase activity in *Mucor hiemalis* (Hiol *et al.*, 1999), *Fusarium solani* (Savitha *et al.*, 2007), *Ophiostoma piliferum* (Brush *et al.*, 1999), *Humicola lanuginose* (Ibrahim and Hayashi, 1987) and *Aspergillus flavus* (Kareem *et al.*, 2017) were recorded at optimum temperature 45°C. It is important to report that the rate of an enzyme-catalyzed reaction increases as the temperature is raised in most chemical reactions. The rise of 1-2°C in temperature of the reaction will only activate 10 to 20% of the enzyme activity, however; a rise of 10°C will jump the activity of most enzymes from 50 to 100%. This will result in the substrate no longer fitting, so the reaction will proceed more slowly. Generally, the reaction rate increases with temperature to a maximum level, then directly decreases with additional increase of temperature. This means that if the temperature rises above or below optimum, the shape of the active site of the enzyme will change, so the enzyme will be less active or even denatured (Harrow and Mazur, 1958).

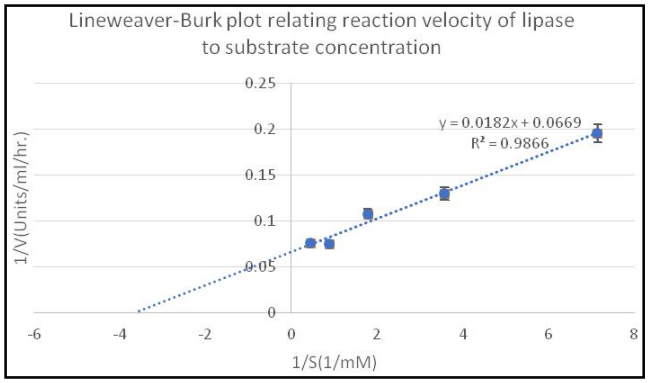

Fig. 5: Lineweaver-Burk plot of lipase activity in *C. catenulate*.

Effect of substrate concentrations on lipase activity

The enzyme kinetics measured a value of Km and Vmax from Lineweaver-Burk plot of lipase activity at optimum pH and temperature using various concentrations of sorbitane monopalmitate substrate. The Km and Vmax values of lipase were found to be 0.271 mM and 14.94 U/ml, respectively when the substrate is sorbitane monopalmitate Fig. 5.

In this case, Kambourova *et al.*, (2003) revealed that the value of Km and Vmax for *Bacillus* sp. J33 lipase were 2.5 mM and 0.4 M/ml/min, respectively using para-nitrophenyl palmitate (pNPL) as substrate, whereas Km and Vmax for *Bacillus Stearothermophilus* lipase were 0.33mM and 188 µm/min/mg, using the same substrate. Also, Tripathi *et al.*, (2014) reported that the Km and Vmax were 3.2 mM and 50 µmol/min/mg, respectively using pNPL substrate, in *Microbacterium* sp. In addition, the Km and Vmax values (1.13 mM and 86.2 µM/min) for the *Rhizomucor miehei* lipase and (1.08 mM and 98.1 µM/min) *Rhizopus oryzae* lipase were recorded using pNPL substrate (Takó *et al.*, 2017). However, Km and Vmax values were 7.15 mM and 7.067 mM/min for *Trichoderma harzianum*; using a Hanes-Wolf plot (Ulker *et al.*, 2011). It is important to clarify that when the concentration of sorbitane monopalmitate increases, lipase becomes saturated with it. As soon as its catalytic site is empty, more sorbitane monopalmitate will be available to bind and undergo reactions. The rate of product formation will now base on the lipase activity itself, and so, adding more substrate will not affect its reaction rate.

Effect of metal ions and inhibitors on lipase activity

It was found that Na^{+}, K^{+}, Ba^{+2} and Fe^{+2} ions inhibited lipase activity, while Zn^{+2} enhanced its activity. Hg^{+2} significantly reduced the activity of lipase with 0.04 U/ml. table 4. Lipase activity was inhibited in presence of 10 mM EDTA and â-mercaptoethanol which indicated that it is a metalloprotein.

It is interesting to mention that *Rhizomucor miehei* and *Rhizopus oryzae* lipases were significantly inactivated with Hg^{+2}, while K^{+} had no effect on the activity (Takó *et al.*, 2017). In case of *Trichoderma harzianum* IDM14D, Ca^{+2} and Mn^{+2} ions increase lipase activity to 25% and 15%, respectively but K^{+} and Cr^{+3} slightly inhibited lipase activity. In addition, it was not affected by other metallic ions (Ulker *et al.*, 2011). Different *Mucor* species obtained similar results (Hiol *et al.*, 1999;

Abbas *et al.,* 2002). Calcium chloride proved to be the strongest lipase inducer in *Aspergillus japonicus* yielding up to 100% relative activity followed by mercury chloride, magnesium chloride and barium chloride. The enzyme activity was not affected by manganese chloride and cobalt chloride (Jayaprakash and Ebenezer, 2012). The metal ions (Na^+, K^+, NH_4^+, Ca^{+2}, Co^{+2}, Mn^{+2}, Ba^{+2}, Mg^{+2} and Fe^{+2}) and EDTA did not impact lipase activity from the yeast, *Cryptococcus* sp. S-2 (Kamini *et al.,* 2000). The impact of metal ions to lipase stability and activity can differ in its variegating role (inhibition and stimulation). Most thermostable lipases need metal ions as co-factors like Na2+, Ca2+, Mg2+, Ni2+, Fe3+, Cu2+ and Zn2+ for better functions and stability (Ishak *et al.,* 2019). They can play either direct action at the catalytic site, or specific action forming complexes between the metal ions and ionized fatty acids in which they changes their solubility and behavior at interfaces (Supakdamrongkul *et al.,* 2010; Quiroga-Roger *et al.,* 2015).

Effect of detergent and organic solvents

The effects of detergents and organic solvents were assayed on the lipase activity purified from *C. catenulata*. The supply of 1% Tween 80 and SDS significantly enhanced lipase activity while Tween 20 and Triton x-100 inhibited its activity table 5. In addition, minimum activity loss was observed with Tween-80 (16.84%) whereas maximum activity loss was with Tween-20 (59.71%). In case of organic solvents, addition of 70% from each of acetone, isopropanol, butanol or methanol, inhibited the lipase activity compared to control without solvents table 5.

Concerning detergents, Hiol *et al.,* (1999) reported that Triton X-100 and Tween 20 reduced lipase activity in *Mucor hiemalis*. The lipase from *Aspergillus tamarii* JGIF06 retained 90.84 % of its activity in the presence of CTAB, while its lowest residual activity was recorded with Tween 20 (71.83 %), compared to 100 % activity shown by the control (without any surfactant) (Das *et al.,* 2016). Takó *et al.,* (2017) mentioned that lipase activity of both *Rhizomucor miehei* and *Rhizopus oryzae* were significantly inactivated with SDS. Contradictory, Costa-Silva *et al.,* (2014) indicated the potentiality of the lipase produced by fungus *Cercospora kikuchii* to tolerate various surfactants like SDS, CTAB and Tween 80. They found that the lipase activity was stable in the presence of Tween 20, Tween 80, SDS and Triton X-100 surfactants and retained 98–100 % of enzymatic activity. The added surfactant serves to solubilize the substrate, and it also serves to carry the substrate to the enzyme's active site or to reorient the substrate into a better position for the enzyme-catalyzed reaction (Lai and O'Connor, 2000). There have been a few studies involving the effect of detergents on lipase activity (Salameh and Wiegel, 2007; Lai and O'Connor, 2000). Generally, detergents have close resemblance to lipase substrates; they form micelles when they exceed their solubility concentrations. Different detergents will influence lipase activity differently. Substrate and detergent interactions with lipases can be complex and unpredictable (Mogensen *et al.,* 2005; Hermoso *et al.,* 1996; Helistö and Korpela, 1998; Misiorowski and Wells, 1974).

Concerning organic solvents, Jayaprakash and Ebenezer, (2012) reported that *Aspergillus japonicus* lipase retained almost 90% activity in presence of acetone, chloroform, methanol, ethanol and hexane except for butanol. Also, there was a gradual decline in enzyme activity, with increased concentration of solvents. Activity without the solvent was set at 100%. In addition, SDS (the anionic surfactant) had an inhibitory effect on lipase activity. In close relation, all solvents exerted an inhibitory effect on lipase activity in *Aspergillus fumigatus* (Mehta

Table 4: Effect of metal ions and inhibitors on lipase purified from *C. catenulate.*

Metal ion (10mM)	Lipase activity (U/ml)	Specific activity (U/mg)
Control	13.23±0.030^h	6.3
NaCl	5.69±0.010^f	2.70
KCl	4.83±0.017^d	2.3
$BaCl_2$	6.55±0.015^g	3.11
$ZnSO_4.7H_2O$	14.60±0.010^i	6.95
$HgCl_2$	0.040±0.010^a	0.01
$FeCl_3$	5.29±0.015^e	2.51
EDTA	4.14±0.015^c	1.97
β- mercaptoethanol	1.60±0.010^b	0.76

P ≤ 0.05; each value represent the mean of triplicates ±SD.

Table 5: Effect of detergent and organic solvents on lipase purified from *C. catenulate.*

Detergent (10mM)	Lipase activity (U/ml)	Specific activity (U/mg)
Control	13.25±0.010^g	6.30
Triton X-100	5.93±0.015^c	2.82
Tween 20	12.78±0.010^f	6.08
Tween 80	13.84±0.015^h	6.59
SDS	19.19±0.015^i	9.13
Acetone	4.63±0.015^b	2.20
Isopropanol	7.37±0.025^e	3.50
Butanol	3.84±0.005^a	1.82
Methanol	6.07±0.020^d	2.89

P ≤ 0.05; each value represent the mean of triplicates ±SD.

et al., 2018). Also, *Aspergillus tamarii* JGIF06 purified lipase was inhibited when treated with 2-propanol, acetone, methanol and ethanol (Das *et al.*, 2016). Lipolytic activity of purified *Rhizomucor miehei*, *Rhizopus oryzae* lipases in the presence of different primary and secondary alcohols and alkanes, was decreased in the presence of 5-10% (v/v) hexanol and butanol and small concentrations of methanol, ethanol, propanol, while isopropanol did not have a major impact on pNPP hydrolysis (Takó *et al.*, 2017). It is interesting to know that the addition of hydrophilic and polar solvents to the enzyme will destabilize it by removing essential water molecules from enzymes. Enzymes are more stable in hydrophobic solvents than hydrophilic solvents as they have less capability to remove the water from the enzyme (Ishak *et al.*, 2019).

Conclusion

The virulent factor lipase in *Candida catenulata* was affected by the presence of antifungals especially in combination treatment. Its purification and characterization indicated that its optimum activity was at pH 5.5 and temperature 37°C and it has K_m and V_{max} values of 0.271 mM and 14.94 U/ml, respectively using sorbitane monopalmitate as substrate. Its activity was inhibited by Na^+, K^+, Ba^+ and Fe^{+2} ions as well as Tween 20 and Triton x-100 detergents. Also, its activity was reduced by Hg^{+2} ions and enhanced by Zn^{+2} ions, in addition to Tween 80 and SDS detergents.

Future perspectives

Lipases like other solubilized enzymes are difficult to recover in the reaction medium. During the process, some parameters (as pH and temperature) may cause its denaturation and so, its loss of activity. Most of the enzymes can maintain their activity for several cycles, which allows their reuse. Their reuse is by their immobilization using substrates insoluble to the reaction medium to give it stability, high conversion rates and activity under extreme conditions.

So, attention must be made to enzymatic immobilization, especially lipases, as a cheap alternative to reduce the limitations of soluble enzymes. In this way, lipases immobilized can be used on the industrial scale.

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