ORIGINAL ARTICLE

Effect of some mutagenic compounds on lipase production by fluconazole-treated *Candida catenulata*

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Abstract

Lipases (triacylglycerol acylhydrolases, E.C. 3.1.1.3) are enzymes widely applied in industry and medicine. Our previous study showed that *Candida catenulata* produced much higher lipase activity in presence of 150 mg fluconazole than that in untreated control. Consequently in this study, optimization of fluconazole-treated *C. catenulata* for maximum lipase production was carried out. Fluconazole-treated *C. catenulate* cultured on medium containing 2.5% Tween 80 as carbon source, 0.7% yeast extract as nitrogen source, and 10 mM $CuSO_4.5H_2O$. pH 5.5, dark incubation at 37 °C under dark agitation for 3 days expressed the optimum physiological variables for maximum lipase production. Transmission electron microscopy (TEM) of the native cell (control) of *C. catenulata* declared a typical morphology of *Candida* cell with a uniform central density, typical structured nucleus, and a cytoplasm with several elements of endomembrane system and enveloped by a regular intact cell wall. The TEM photomicrograph of 150 mg fluconazole-treated cells of *C. catenulata* showed that the ultrastructures of treated *C. catenulata* displayed many noticeable morphological changes. The effect of mutagens [sodium azide (SA), ethidium bromide (EtBr), ethanol (EtOH)] was assayed on the lipase activity from *C. catenulata*. All mutagenic compounds with different concentrations decreased the lipase activity. Yeast specific activity, protein concentrations, and dry biomass gradually reached their highest decline at mutagen-elevated concentrations. Combination of lower concentrations of mutagen (0.1 µg/ml EtBr + 0.5 mM SA + 10% EtOH, Comb-Fl) exerted synergistic inhibition action and reduced *C. catenulata* virulent factor (lipase) over their single treatment. When 150 mg fluconazole was added to the mutagen's combination mixture (Comb + Fl), an increase in the production of the virulence factor lipase by *C. catenulata* was detected. This action indicated the antagonistic effect of fluconazole over the mutagen combination mixture in increasing the fungal pathogenicity.

Keywords *Candida catenulata* · Fluconazole · Lipase · Mutagens · Ultrastructures

1 Introduction

Lipases (E.C. 3.1.1.3) belong to a class of hydrolases and water-soluble enzymes that catalyze insoluble triacylglycerol hydrolysis to produce free fatty acids, diacylglycerols, monoacylglycerols, and even glycerol. They catalyze a whole range of reactions like hydrolysis, transesterification, and interesterification of other esters and ester synthesis, and display a range of regioselective, enantioselective, and stereoselective transformation properties [28, 71].

Lipases are present everywhere and from different sources but microbial lipases are the most commonly ones used in modern industrial processes such as detergent formulations, synthesis of biosurfactant, dairy formation, paper, cosmetics, and pharmaceuticals [33, 36, 83]. The microbial lipases are extracted industrially, much more than that of the plants and animals. Microbes have short generation cycle, pervasive nature, and great metabolic divergence so that the enzymes can be isolated easily [28]. Microbial lipases also have abilities to work at high temperatures, pH, and stable in organic solvents, chemoselective, regioselective, and enantioselective. In addition, they have higher output yields, ease of use genetic engineering, and rapid growth on cheap media, allowing more suitable production [77]. The most frequently utilized fungal genera in those applications are *Penicillium*, Mucor, *Geotrichum*, *Rhizopus*, and *Aspergillus* [3, 14, 29, 48]. Numerous species of *Candida* genus had also

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an extracellular ability to hydrolyze and synthesize various oils [5, 11, 35, 38, 68, 74], Alami et al. [4]. This ability gives them great prospects in lipase-producing industries [16].

Component of the medium composition greatly influences production of lipase especially carbon sources (lactose, glucose, and galactose), nitrogen (yeast extract, urea, ammonium nitrate, peptone, corn steep liquor, and amino acids), and surfactants as Tween 20 and Tween 80 [1, 19, 20]. In the published literatures, some investigators studied the effect of medium components and conditions on lipase activities of *Candida* spp. such as Tsuboi et al. [80], Jatta et al. [38], Ali et al. [5], Salihu et al. [68], Boonchaidung et al. [11], and Alami et al. [4], but none was recorded on *C. catenulata*.

Popular therapies for invasive candidiasis and mucocutaneous infections depend on some compounds such as vulvovaginal candidiasis (VVC) including polyene (nystatin, amphotericin B, and its formulations of lipids), azoles (fluconazole and voriconazole), and echinocandins (caspofungin) [59]. The azoles, as fluconazole, are one of the most popular antifungals, and also have a direct impact on *Candida* sp. morphology and hyphae development [34]. The impact of azole-mediated hyphal repression is important enough that hyphal branching is limited at subinhibitory azole concentrations and that the yeast-to-hyphal transformation is fully prevented at clinically significant doses [34]. It was announced that *Candida albicans* cells treated with azoles developed higher farnesol levels [37]. A fungal-produced quorum sensing agent is capable of preventing the development of filaments in *C. albicans*. Azoles target enzymes involved in sterol biosynthesis and azole treatment contributes to the formation of the sterol biosynthetic intermediate, farnesyl pyrophosphate, which indirectly promotes the overproduction of farnesol [7, 37].

Mutagenesis, either by chemical or physical agents, is one of the preferred methods for strain improvement to achieve maximum enzyme productivity in microbes. Several investigations mentioned to use this technique to improve lipase activity in mutants as *Aspergillus fumigatus* MTCC 9657 [64], *Aspergillus japonicas* [44], *Aspergillus niger* [79], *Candida rugosa* [60], and *Rhizopus* sp. BTS-24 [8]. Similarly, others reported mutagenic methods to enhance protease activity in mutants as *Aspergillus niger* [62] and *Trichoderma harzianum* T334 [75].

From previous study [35], we found that *Candida catenulata* produce high lipase activity (20.31 U/ml) in presence of fluconazole (than other antifungal drugs), so the current study aimed to optimize the fluconazole-treated *C. catenulata* in presence of variable factors to maximize the extracellular lipase production then to impact some mutagens with/without fluconazole on the lipase activity and on the cellular structures of *C. catenulata*.

2 Materials and methods

2.1 Test organism

Candida catenulata was kindly provided by Micro Analytical Center, Microbiology Lab, Cairo University. This species was isolated from blood sample in Abu Al-Rish Japanese Children's Hospital.

2.2 Chemicals

Flucoral (SEDICO) containing 150 mg fluconazole was used as systemic antifungal agents. Sodium azide (SA) and ethidium bromide (EtBr) were purchased from ICN Biomedicals Inc., while ethanol (EtOH) was HPLC grade and was used as mutagens.

2.3 Culturing and maintenance

Candida catenulata was maintained in medium composed of (%): peptone (1), glucose (2), and agar (3) and incubated at 37 °C for 48 h. It was subcultured according to Bramono et al. [12] and kept in refrigerator until use.

2.4 Lipase extraction and assay

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 [80]. Fifty milliliters of the culture medium contained in a 200-ml flask was kept at 37 °C for 20 min 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 sorbitan monopalmitate (modified from alpha-naphthylpalmitate), 14 mM of citric acid, and 74 mM of Tris buffer (pH 5.5) [for 1 h] 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 1 μmol of substrate (sorbitan monopalmitate in the present study) per minute [80].

2.5 Specific activity

It is the activity of an enzyme per milligram of total protein (expressed in $\mu mol\ min^{-1}\ mg^{-1}$) or (U/mg). Specific activity measures the enzyme purity in mixture. It is the micromoles

of product formed by an enzyme in a certain time (minutes) under specific conditions per milligram of total proteins. The precipitates of organism were used to estimate the dry biomass in each experiment [35].

2.6 Biomass

Dry biomass (g/l) was determined after drying the *C. catenulata* cells at 60 °C until constant weight.

2.7 Determination of protein concentration

The concentration of proteins was determined according to the method mentioned by Lowry et al. [50]. One milliliter of the enzyme preparation was added to a mixture which consisted of 0.1 ml of 4% NaOH, 0.5 ml of 2% Na_2CO_3, and 0.5 ml of freshly prepared solution [1% CuSO4 and 2% potassium sodium tartrate (1:1 v/v)]. They were well mixed and left for 10 min at room temperature. Thereafter, 0.1 ml of Folin-Ciocalteu reagent was added to the mixture and shaken well. After 30 min., the optical density was measured spectrophotometrically at 750 nm. Bovine serum albumin standard curve was used to estimate the protein concentrations (mg/ml).

2.8 Optimization of growth factors

Eight factors were studied to elucidate the optimum medium for maximizing lipase production and activity by *C. catenulata*. The effect of different media was carried out using Czapek Dox's medium, Sabouraud medium, and Tsuboi et al. [80] medium (0.7% yeast extract + 2.5% Tween 80). Carbon sources of 2.5% sucrose, maltose, glucose, and Tween 80 were tested. Nitrogen sources (yeast extract, peptone, sodium nitrate, and ammonium sulfate) at 0.7% were used. The impact of metal ions, at concentration of 10 mM, of $ZnSO_4.7H_2O$, $(NH4)_2SO4$, $CuSO4.5H_2O$, and $MnSO_4.7H_2O$ were assessed. Before medium sterilization, various pH values (4, 4.5, 5, 5.5, and 6) were adjusted for investigation. Different agitation were assayed in different incubator conditions as light static, dark static, light shaking, and dark shaking.

Different temperatures (25, 30, 35, 37, and 40 °C) and different incubation periods (1, 2, 3, and 4 days of incubation) were also tested. In all cases, the optimum factor for lipase production from each experiment was used in the next one. Lipase assay was estimated in culture filtrate after growth of *C. catenulata* disks in each experiment.

2.9 Cytological changes exerted by the most potent treatment on *C. catenulata* cells

The transmission electron microscopy (TEM) technique was performed at the Regional Center for Mycology and

Biotechnology (RCMB), Al-Azhar University, Cairo, Egypt. Both untreated (control) and treated *C. catenulata* cells were collected separately by centrifugation (at 4000 rpm for 10 min) from 72-h-old cultures grown on liquid medium containing 0.7% yeast extract (modified from yeast nitrogen base) and 2.5% Tween 80 for lipase induction [80]. After washing with distilled water, the samples were prepared for examination by TEM using the procedures described below:

Specimens were fixed by immersing in freshly prepared aqueous potassium permanganate solution (1% w/v) for 5 min at room temperature. Fixed specimens were then washed three times by distilled water for 15 min, and then they were dehydrated in ethanol graded series each for 15 min. The absolute alcohol was replaced with acetone via a stepwise series of ethanol:acetone (2:1, 1:1, and 1:2 v/v), and then finally maintained in water-free acetone for an hour. Dehydrated specimens were infiltrated with the resin mixture through a graded series of resin:acetone and then in pure resin at 60 °C for 72 h then allowed to attain room temperature.

The dehydrated specimens fixed with potassium permanganate were embedded in blocks filled with epoxy resin mixture overnight in an oven at 60 °C then return to room temperature. Using a stereomicroscope (Leica), the mounted blocks were trimmed with razor blades to give a trapezoid-shaped face of less than 1 mm width and height. Ultra-thin sections were cut with a Leica ultracut (UCT) ultramicrotome and the sections (straight ribbon) were manipulated to picking up on grids.

The grids of copper hexagonal (2.05 mm mesh) were degreased and the sections were fastened to clean glass slide adhesive prior to staining. Sections were stained with double stain (uranyl acetate followed by lead citrate) according to Reynolds [67]. Stained sections were examined with transmission electron microscope [JEOL (JEM 1010)] at different magnifications. The images were captured by a built-in camera.

2.10 Mutagenesis of *Candida catenulata* and its effect on lipase enzyme activity

In the first experiment, the mutagens used to induce mutation on *Candida catenulata* were ethidium bromide (EtBr), sodium azide (SA), and ethanol (EtOH), and they were added singly. Their concentrations were 0.1, 0.3, and 0.5 μg/ml of ethidium bromide, 0.5, 1, and 2 mM of sodium azide, and 10, 30, and 70% of ethanol.

Four disks of *C. catenulata* were transferred to 20 ml of each mutagen (type and conc), shake carefully, and incubated for 1 h at room temperature. After incubation, the samples were centrifuged at 4000 rpm. The precipitates were transferred to 20 ml subculture liquid medium (1% peptone + 2% glucose) to make suspensions. Two

milliliters of suspension was added to triplicate flasks, and each contains 60 ml of lipase medium (0.7% yeast extract + 2.5% Tween 80) and then incubated for 3 days at 37 °C. After incubation, the samples were centrifuged at 4000 rpm. Untreated *C. catenulata* was represented as control and was prepared similarly but without prior incubation.

The best concentration (with high lipase activity) from each mutagen treatment (of the previous experiment) was taken in combination, representing 0.1 µg/ml EtBr, 0.5 mM SA, and 10% EtOH singly or in combination with flucoral (150 mg fluconazole). Similarly, the previous experimental steps were carried out using optimized lipase medium.

In all cases, the filtrates were collected for lipase activities, protein concentrations, and specific activities. The cells were collected for TEM studies. All mutagen work was carried out under aseptic conditions in a laminar airflow cabinet.

2.11 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.

3 Results and discussion

3.1 Effect of different media

Table 1 indicates that lipase produced from *C. catenulata* achieved its maximum activity in medium composed of 0.7% yeast extract + 2.5% Tween 80 with enzyme activity of 20.498 U/ml, protein content of 2.48 mg/ml, and specific activity of 8.26 U/mg. In this medium, the dry biomass recorded the highest gain with 0.146 g/l. In agreement with this previous report, the investigated medium 0.7% yeast extract + 2.5% Tween 80 induced high *Candida* sp. lipase [80]. Minimum lipase activity was attained in a glucose yeast extract medium [81]. However, in *Pseudomonas* sp., glucose yeast extract peptone (GYP) was the optimum medium for lipase production [70].

3.2 Effect of carbon source

Table 2 reveals that maximum lipase production occurred in presence of Tween 80 with enzyme activity of 20.90 U/ml and specific activity of 8.42 U/mg. Sucrose also enhanced lipase production with activity and specific activity of 4.69 U/ml and 1.39 U/mg, respectively. Meanwhile, other carbon sources inhibited lipase activity. Tween substrates have a good inductive effect on the production of fungal lipase [46]. A linear increase in lipolytic activity was shown in presence of Tween 80 on

Table 1 Effect of different media on lipase activity, protein concentration, specific activity, and dry biomass gain of *C. catenulata*

Type of medium	Lipase activity (U/ml)	Protein conc. (mg/ml)	Specific activity (U/mg)	Dry biomass (g/l)
Czapek Dox's	1.20 ± 0.145^a	6.71 ± 0.141^c	0.17	0.09 ± 0.005^a
Sabouraud dextrose	1.12 ± 0.121^a	4.41 ± 0.020^b	0.25	0.09 ± 0.010^a
0.7% yeast extract + 2.5% Tween 80	20.50 ± 0.012^b	2.48 ± 0.011^a	8.26	0.15 ± 0.005^b

Values followed by dissimilar letters within columns are significantly different ($p \leq 0.05$); each value represents the mean of triplicates ± SD

Table 2 Effect of carbon source on lipase activity, protein concentration, specific activity, and dry biomass gain of *C. catenulata*

Carbon source	Lipase activity (U/ml)	Protein conc. (mg/ml)	Specific activity (U/mg)	Dry biomass (g/l)
Control	0.41 ± 0.077^a	4.07 ± 0.076^c	0.101	0.04 ± 0.00^a
Tween 80	20.90 ± 0.015^e	2.48 ± 0.010^a	8.42	0.15 ± 0.005^c
Sucrose	4.69 ± 0.121^d	3.36 ± 0.010^b	1.39	0.14 ± 0.017^c
Maltose	1.45 ± 0.076^b	4.14 ± 0.155^c	0.350	0.06 ± 0.010^a
Glucose	2.18 ± 0.170^c	3.44 ± 0.152^b	0.633	0.10 ± 0.015^b

Values followed by dissimilar letters within columns are significantly different ($p \leq 0.05$); each value represents the mean of triplicates ± SD

growth medium of *Mortierella vinace* [30]. Cihangir and Sarikaya [17] suggested that *Aspergillus niger* grown on carbohydrate-supported media achieved lower lipolytic activity compared to oil as the sole substrate. Considering the dry biomass, Tween 80 recorded the highest value (0.146 g) compared to control and other sugars. Similarly, the main influencing factor for the growth of fungal lipase was oil as a carbon source [10].

3.3 Effect of nitrogen source

Maximum lipase production was obtained in presence of yeast extract as nitrogen source with activity of 21.0 U/ml and specific activity of 8.50 U/mg. This was followed by peptone and $NaNO_3$, and minimum activity was recorded with $(NH_4)_2SO_4$. Higher dry biomass gain of 0.148 g/l was achieved also in presence of yeast extract as nitrogen source in *C. catenulata* medium (Table 3). Yeast extract was very efficient in the production of lipase by the yeast *Cryptococcus*, spS-2 with activity of 26.0 U/ml [42], with 25.0 U/ml in *Aspergillus niger* [18, 26], and with 1.134 U/ml in *Candida* sp. KKU-PH2-15 [11]. Among the various nitrogen sources added to the modified glucose-yeast-peptone (GYP) medium, only NH_4NO_3, KNO_3 enhanced lipase production, while the other inorganic nitrogen sources had an inhibitory effect on the production of lipase by *Pseudomonas* sp. [70].

3.4 Effect of metal ions on lipase production

It was observed that Cu^{+2} promoted the activity of lipase followed in descending order by Zn^{+2} and Mn^{+2} (Table 4). The highest lipase activity, specific activity, and biomass gain of 22.95 U/ml, 9.14 U/mg, and 0.154 g/l, respectively, were attained in presence of Cu^{+2} in the growth medium of *C. catenulata*. Maximum lipase activity in *Lactobacillus delbrueckii* subsp. *bulgaricus* was achieved with the addition of 0.01 M of $CaCl_2$ [27]. The addition of 2% Ca^{+2} in production medium resulted in maximum lipase production, whereas the other metal ions decreased lipase production. The transition metals, especially Ni^{+2}, led to decrease in activity of up to 88%, with somewhat lesser reductions related with Mg^{+2}, Na^+, K^+, and Cu^{+2} [69]. This negative effect of metal ions was reviewed by Joseph et al. [40].

3.5 Effect of pH

pH has an impact on ionization of active sites involved in substrate binding and catalysis [21]. In this study, it was found that the highest lipase activity and specific activity were obtained at pH 5.5 with 23.1 U/ml and 9.31 U/mg, respectively (Fig. 1). Also, at pH 5.5, the highest dry biomass of 0.156 g/l was recorded. Any change in this pH value led to decrease in enzyme production. In previous reports, maximum lipase development of *Cryptococcus* spS-2 at pH 5.6 has been reported, with activity of 65.7 U/ml [42]. The peak of lipase activity was observed when the initial medium

Table 3 Effect of nitrogen source on lipase activity, protein concentration, specific activity, and dry biomass gain of *C. catenulata*

Nitrogen source	Lipase activity (U/ml)	Protein conc. (mg/ml)	Specific activity (U/mg)	Dry biomass (g/l)
Control	0.72 ± 0.096^a	1.98 ± 0.020^a	0.36	0.04 ± 0.00^a
Yeast extract	21.00 ± 0.015^e	2.47 ± 0.015^d	8.50	0.15 ± 0.005^e
Peptone	14.96 ± 0.265^d	2.21 ± 0.011^c	6.76	0.13 ± 0.003^d
$NaNO_3$	11.18 ± 0.210^c	2.01 ± 0.015^b	5.56	0.11 ± 0.011^c
$(NH4)_2SO_4$	10.65 ± 0.127^b	1.96 ± 0.015^a	5.43	0.07 ± 0.015^b

Values followed by dissimilar letters within columns are significantly different ($p \leq 0.05$); each value represents the mean of triplicates $\pm$ SD
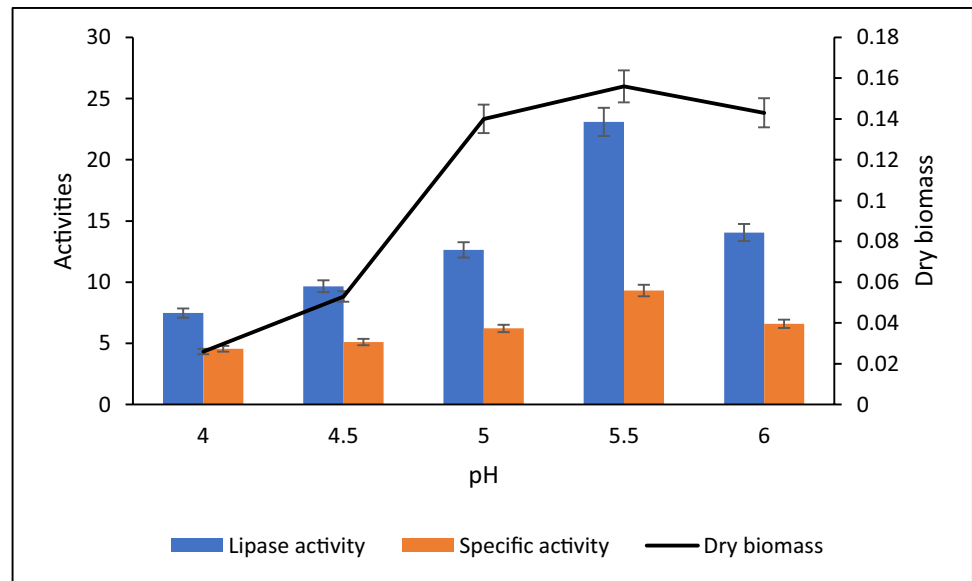
Table 4 Effect of metal ions on lipase activity, protein concentration, specific activity, and dry biomass gain of *C. catenulata*

Metal ions	Lipase activity (U/ml)	Protein conc. (mg/ml)	Specific activity (U/mg)	Dry biomass (g/l)
Control	$21.20 \pm 0.003c$	2.48 ± 0.010^d	8.54	0.15 ± 0.001^a
$(NH4^+)$	9.33 ± 0.202^a	2.30 ± 0.005^a	4.05	0.14 ± 0.034^a
(Zn^{+2})	12.05 ± 0.480^b	2.43 ± 0.017^c	4.95	0.15 ± 0.025^a
(Cu^{+2})	22.95 ± 0.012^d	2.51 ± 0.00^d	9.14	0.15 ± 0.005^a
(Mn^{+2})	12.32 ± 0.265^b	2.36 ± 0.030^b	5.22	0.14 ± 0.005^a

Values followed by dissimilar letters within columns are significantly different ($p \leq 0.05$); each value represents the mean of triplicates $\pm$ SD

Fig. 1 Effect of pH on lipase activity, specific activity, and dry biomass from *C. catenulata*


pH of *Pseudomonas* sp. was adjusted at 5.5 [70]. *Penicillium roqueforti* has achieved optimum lipase yield at pH 5.5 [52]. Lin et al. [49] and Rehman et al. [66] stated that pH 5.5 in *Penicillium notatum* and *Antrodia cinnamomea* was the optimal for lipase production. In contrary to ours, Jatta et al. [38] found that optimum pH for the lipase activity of *C. albicans* was 7 when grown on both Sabouraud dextrose broth (1600 U/mg) and Lee synthetic medium (200 U/mg).

3.6 Effect of temperature

Optimum temperature was found to be 37 °C with lipase activity and specific activity of 23.3 U/ml and 9.39 U/mg, respectively (Fig. 2). The gradual increase in temperature

led to concomitant increase in lipase activity while higher temperature above the optimum caused a significant reduction in enzyme activity. At 37 °C, the highest dry biomass of *Candida catenulata* had been recorded with gain of 0.157 g/l. Jatta et al. [38] found that optimum temperature for the lipase activity of *C. albicans* was 37 °C when it is grown on both Sabouraud dextrose broth and Lee synthetic media. Maximum yeast lipase development from *Cryptococcus* spS-2 was achieved at temperature 25 °C [42]. Lipase activity in *Pseudomonas* sp. was maximum at 37 °C [70]. The *Rhodotorula glutinis* [22] and *Penicillium citrinium* [53] have optimum temperature at 30 °C and 22 °C, respectively, whereas maximum lipolytic activity of *Trichoderma harzianum* occurred at temperature 40 °C [81].

Fig. 2 Effect of temperature on lipase activity, specific activity, and dry biomass from *C. catenulata*
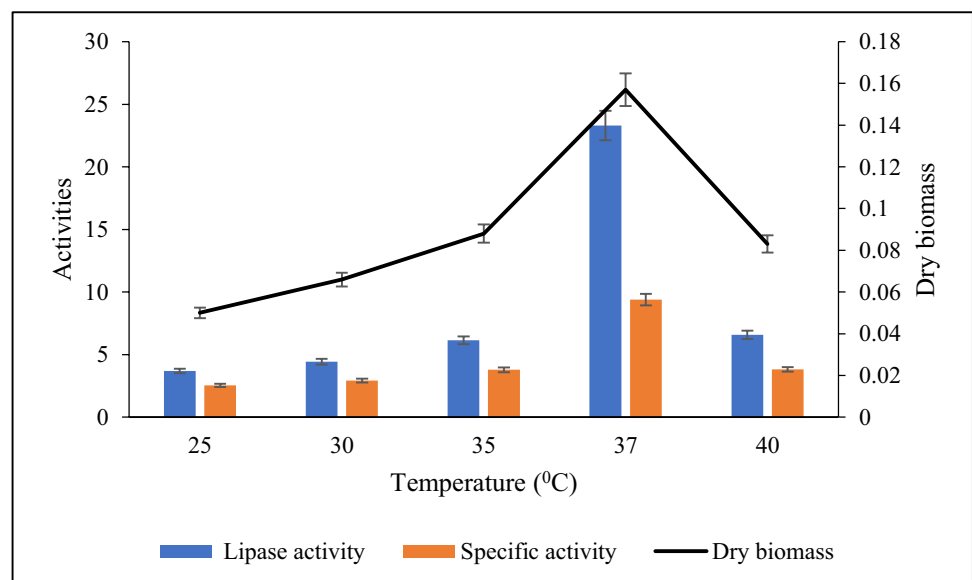
3.7 Effect of light and agitation

Agitation is a significant variable that enhances the growth and lipase development by fungi. It enhances the rate of oxygen transfer and thus increasing the dispersal of oil micelles for greater interaction with fungal cells, and enhances the absorption of oil that induces lipase secretion [76]. In the present study, maximum lipase production and biomass gain by *C. catenulata* were obtained at dark agitated condition with 23.9 U/ml and 0.160 g/l, respectively (Table 5) followed by light static with activity of 17.34 U/ml and 0.157 g/l, respectively. *Bacillus sudeticus* showed that the activity of lipase on agitated culture was fourfold higher than non-agitated culture [85]. Activity of lipase in *B. ribis* also reported a 98% increase in agitated culture relative to static ones [55]. Similarly, *Aspergillus* sp. detected significant enhancement in lipase secretion in agitated condition [17]. A considerable lipase activity in the modified GYP medium of *Pseudomonas* sp. was obtained in shaking condition [70]. Also, Velmurugan et al. [82] reported that maximum fungal biomass development was obtained in darkness condition, and minimal production was obtained under the yellow light in five filamentous fungi (*Monascus purpureus*, *Isaria farinosa*, *Emericella nidulans*, *Fusarium verticillioides*, and *Penicillium purpurogenum*).

3.8 Effect of incubation period

It was observed that lipase activity and biomass yield were increased to record the highest value at the 3rd day of incubation with activity of 24.1 U/ml and weight of 0.11 g/l. Further increase in incubation period above the optimum led to decrease in both parameters (Fig. 3). Similarly, *Candida* sp. developed the highest lipase activity on the 3rd day of incubation [80]. In the same context, *Pseudomonas* sp., incubation time for peak lipase activity was 3 days [70]. *Pseudomonas flourescens* reported optimum lipase activity after 24 h [43], while maximum lipase activity (25 U/ml) was observed after 72 h of incubation time in *A. niger* [26].

3.9 Mutagenesis of *Candida catenulata* and its effect on lipase enzyme activity

The effect of mutagens was assayed on the lipase activity from *C. catenulata* (Table 6). All mutagen types and concentrations decreased the lipase activity, its specific activity,

Table 5 Effect of different light and agitation states on lipase activity, protein concentration, specific activity, and dry biomass gain of *C. catenulata*

Light and agitation state	Lipase activity (U/ml)	Protein conc. (mg/ml)	Specific activity (U/mg)	Dry biomass (g/l)
Light static	17.34 ± 0.076^c	2.48 ± 0.010^c	6.99	0.16 ± 0.003^c
Dark static	9.35 ± 0.174^b	2.12 ± 0.010^b	4.41	0.14 ± 0.005^b
Light shaker	7.97 ± 0.230^a	2.04 ± 0.005^a	3.90	0.10 ± 0.005^a
Dark shaker	23.90 ± 0.013^d	2.50 ± 0.005^d	9.56	0.16 ± 0.002^c

Values followed by dissimilar letters within columns are significantly different ($p \leq 0.05$); each value represents the mean of triplicates $\pm$ SD

Fig. 3 Effect of incubation period on lipase activity, specific activity, and dry biomass from *C. catenulata*
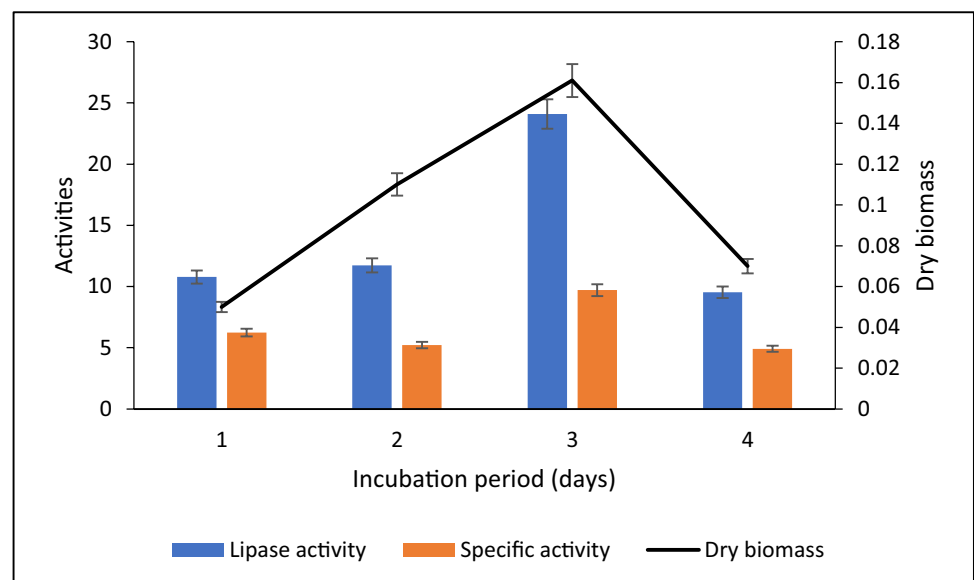
Table 6 Assay of lipase activity, protein concentration, specific activity, and dry biomass of untreated and treated *Candida catenulata* with different concentrations of either ethidium bromide, sodium azide, or ethanol

Mutagen agents	Concentrations	Lipase activity (U/ml)	Protein conc. (mg/ml)	Specific activity (U/mg)	Dry biomass (g/l)
Control (no mutagen)		13.01 ± 0.485[e]	1.95 ± 0.010 [g]	6.67	0.08 ± 0.001 [g]
Ethidium bromide (EtBr)	0.1 μg/ml	7.07 ± 2.17[b]	1.77 ± 0.025[f]	3.99	0.07 ± 0.002[f]
	0.3 μg/ml	6.44 ± 0.098[b]	1.76 ± 0.010[ef]	3.65	0.07 ± 0.001[ef]
	0.5 μg /ml	4.64 ± 0.273[a]	1.73 ± 0.005[de]	2.68	0.05 ± 0.002[a]
Sodium azide (SA)	0.5 mM	11.04 ± 0.132[d]	1.76 ± 0.010[ef]	6.27	0.07 ± 0.003[f]
	1 mM	9.85 ± 0.655 [cd]	1.71 ± 0.015 [cd]	5.76	0.06 ± 0.001[de]
	2 mM	6.55 ± 0.174[b]	1.60 ± 0.015[a]	4.09	0.06 ± 0.0005[c]
Ethanol (EtOH)	10%	11.20 ± 0.147[d]	1.70 ± 0.015[c]	6.58	0.06 ± 0.010 [cd]
	30%	10.22 ± 0.140 [cd]	1.66 ± 0.015[b]	6.15	0.05 ± 0.002[bc]
	70%	9.29 ± 0.147[c]	1.64 ± 0.010[b]	5.66	0.05 ± 0.001[ab]

The values are the mean of triplicates ± SD; dissimilar letters within columns are significantly different ($p \leq 0.05$)

protein concentrations, and dry biomass gradually reaching their maximum decline at mutagen higher concentrations. Lipase activity, specific activity, and protein concentrations of control (no mutagen) still recorded the highest values 13.01 U/ml, 6.67 U/mg, and 1.95 mg, respectively. EtBr declined the lipase activity by about 50% (at 0.1 and 0.3 μg/ml) to more than 60% (at 0.5 μg/ml) compared to control. Lipase specific activity showed similar declined trend. However, the protein concentrations were significantly unchanged by increasing EtBr concentrations, but still lower than the control by about 0.1 folds. SA also decreased the lipase activity but not as sharp as EtBr. Lipase activity declined by 15% (at 0.5 mM SA), then 25% (at 1 mM SA), and finally by 50% (at 2 mM SA). Lipase specific activities were inversely proportional to SA concentrations. Protein concentrations showed similar declined trend. EtOH exhibited less harmful effect on lipase activity (as well as its specific activity) as compared to both EtBr and SA. EtOH decreased lipase activity by 28% and its specific activity by 15% of the value of control at higher concentration (70%). Protein concentrations were insignificantly decreased at higher EtOH concentrations (30 and 70%), while dry biomass decreased by all treatments over the control.

Mutagenesis, either by chemical or physical agents, is one of the methods used for strain improvement to achieve maximum enzyme productivity in microbes. Several investigations mentioned to use this technique to improve lipase activity in mutants as *Aspergillus fumigatus* MTCC 9657 [64], *Aspergillus japonicas* [44], *Aspergillus niger* [79], *Candida rugosa* [60], and *Rhizopus* sp. BTS-24 [8]. Those results were in contrary with ours as the mutant-derived strains from either mutagen treatment reduced the lipase and specific activity as compared with the wild strain. It is interesting to mention that random point mutations may cause silent, nonsense, or missense mutations. Thus, those

reduced activities may be due to missense point mutations that alter the gene sequence, change the amino acids of the enzyme, and so reduce (non-conservative) its activity [63]. It is also important to have a glimpse on the mode of action of the used mutagens: The mutagenicity of SA is accomplished through the production of an organic metabolite of SA [58] which first enters in the cell nucleus, interacts with DNA, and induces point mutation in the host genome [45]. The azide ion alters the structure of cytosine such that it forms hydrogen bonds with adenine, rather than guanine. This produces a cytosine to thymine transition [39]. However, EtBr is intercalating dye that form complexes with double-strand ds-DNA and single-strand DNA by forming close van der Walls contacts with the adenine–thymine base pairs. It also binds to the hydrophobic interior of the DNA molecule causing an increase in the distance between adjacent base pairs and so mutation of DNA [13]. Ethanol is a hydrophilic polar solvent that can create homogenous systems with water, so strip off essential water molecules from the enzyme and easily penetrate its surface causing its denaturation and losing its activity [25]. It also acts as mutagen via its first metabolite, acetaldehyde which stimulates chromosomal aberrations, sister-chromatid exchanges, and cross-links between DNA strands. It also impedes RNA formation in cells and in cell-free transcriptional systems [57].

In addition, dry biomass and protein concentrations decreased by all mutagen treatments over the control. The reduction of growth exerted by SA may be attributed to the decrease in ATP accessibility of the cell which may retard the cell division of the microbe by (1) interacting with enzyme activities and DNA in the cell [54], (2) altering the mitochondrial membrane potential [86], and (3) decreasing the cellular Ca-binding protein, which inhibit the proton pump [23]. EtBr affect the membrane constructions, slow cell division, and hold ATP biosynthesis by

(1) interrupting the metabolism and protein synthesis and (2) inhibiting the formation of mitochondrial macromolecules by reducing the number of mitochondrial DNA [47]. Ethanol as hydrophilic polar solvent strips off essential water molecules from hydrophilic proteins of membranes causing their denaturation and losing their activities [25] including their ability to divide. This declined phenomenon on protein concentrations and growth was also observed by some investigators studying mutant stains. Protein synthesis in yeast protoplast is completely inhibited 3 min after SA addition to a final concentration of 10^{-3} M [6]. Survival rate of *Saccharomyces boulardii* was decreased by increasing concentration of EtBr [2]. Growth of *Escherichia coli* was partially inhibited by 1.2×10^{-4} M EtBr, a phenanthridinium trypanocide. *Bacillus cereus* was extremely sensitive to the growth-inhibitory action of ethidium (10^{-5} M) and morphological changes were observed [78]. Treating *Candida albicans* with 2% EtOH caused a 50% inhibition of germ tube induction, while doubling the EtOH concentration at all times triggered 80% inhibition of germ tube induction [15].

The data in Table 7 revealed that combination of lower concentrations of each mutagen (0.1 μg/ml EtBr + 0.5 mM SA + 10% EtOH, Comb-Fl) decreased lipase activity by exerting synergistic inhibition action. This action reduces *C. catenulata* virulence over their single impact on lipase

activity, protein concentration, specific activity, and dry weight recording 6.61 U/ml, 1.63 mg, 4.05 U/mg, and 0.054 g/l.

When 150 mg fluconazole was added to the mutagen combination (Comb + Fl), an increase in the production of the virulence factor lipase by *C. catenulata* (15.68 U/ml) was recorded with specific activity of 8.25 U/mg. This stimulation action indicated the antagonistic effect of fluconazole over the action of mutagens as well as the effectiveness of this new combination on increasing fungal pathogenicity.

The combination of mutagens with fluconazole decreased sharply the dry biomass over the Comb-Fl and the control (Table 7). This may be due to the cumulative stress produced by fluconazole over those produced on mutant derived by mutagen combinations. It was previously mentioned that fluconazole damages cell divisions and destabilizes the fungal cell membrane [72] causing cell content leakage, lysis, and eventual death [31].

The impact of fluconazole and mutagen combinations on lipase, specific activity, and protein concentrations is illustrated in Fig. 4. Results demonstrated that the highest lipase activity was recorded on optimum medium with fluconazole (Opt + Fl) followed by optimum medium without fluconazole (Opt-Fl, control) = optimum medium with both mutagen combination and fluconazole (Opt + Comb + Fl). The least value was estimated on the optimum medium

Table 7 Assay of lipase activity, protein content, specific activity, and dry biomass of treated *Candida catenulata* with combination of ethidium bromide, sodium azide, and ethanol and/or fluconazole

Mutagen agents	Lipase activity (U/ml)	Protein conc. (mg/ml)	Specific activity (U/mg)	Dry biomass (g/l)
Control (no mutagen)	13.01 ± 0.485^b	1.95 ± 0.010^b	6.67	0.08 ± 0.001^c
0.1 μg/ml EtBr + 0.5 mM SA + 10% EtOH (Comb-Fl)	6.61 ± 0.056^a	1.63 ± 0.047^a	4.05	0.05 ± 0.003^b
0.1 μg/ml EtBr + 0.5 mM SA + 10% EtOH + 150 mg fluconazole (Comb + Fl)	15.68 ± 0.035^c	1.90 ± 0.050^b	8.25	0.05 ± 0.004^a

The values are the mean of triplicates ± SD; dissimilar letters within columns are significantly different ($p \leq 0.05$)

Fig. 4 Effect of optimum medium (Opt) alone or in addition to mutagen combination (Comb) and/or fluconazole (Fl) on lipase, specific activity, and protein concentrations
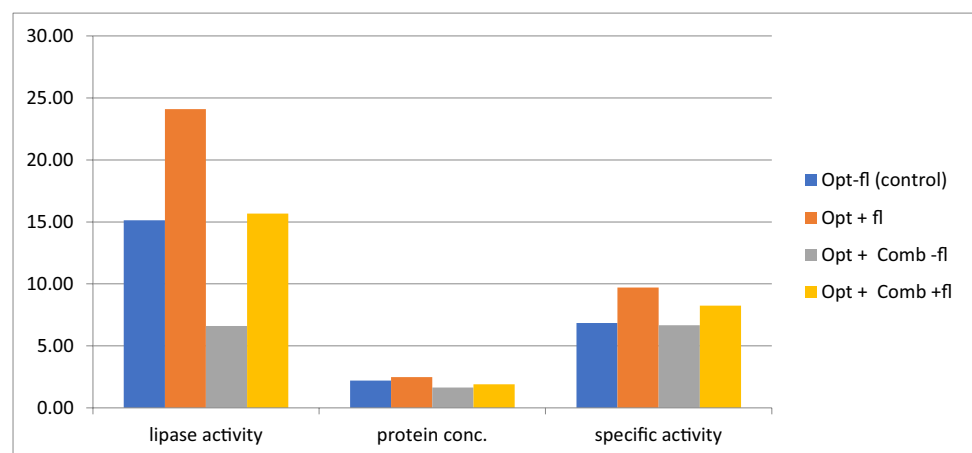
with mutagen combination alone (Opt+Comb-Fl). Similar trend was observed with the specific activity and protein concentrations.

3.10 Examination of the cytological changes exerted by fluconazole treatment on *C. catenulata* cells using transmission electron microscope

The TEM photomicrograph of the control cells (untreated) of *C. catenulata* is shown in Fig. 5. It showed a typical morphology of *Candida* with a uniform central density, a typically structured nucleus, and a cytoplasm with several elements of endomembrane system and enveloped by a regular, intact cell wall as seen in Fig. 5a and b. Control culture showed yeast form and budding cells where cell and chromatin constrict and the cells were spherical to elongate with smooth surface (Fig. 5c-e). Untreated cells of *C. catenulata* presented a normal cell ultrastructure, such as compact and electron-dense cell wall, cytoplasmic membrane, homogeneous and electron-dense cytoplasm, nucleus, and mitochondria.

The typical ultrastructure of *C. catenulata* was not described in the available literatures. In this context, it may be mentioned that some other *Candida* spp. were ultrastructurally described as *C. tropicalis* [24], *C. krusei* [41], and *C. albicans* [9, 56].

The TEM photomicrograph of the treated cells of *C. catenulata* with 150 mg fluconazole is illustrated in Fig. 6.

It was shown that the ultrastructures of treated *C. catenulata* displayed noticeable morphological changes.

Fluconazole treatments caused deformation in cell shape, disintegration of protoplasm, and disruption in the cell wall as well as the cytoplasmic membrane of the cells which led to the protoplasm leakage along with vacuolations as seen in Fig. 6a, d, e, g, h. Most cells were irregular and even hollow to form ghost cells where fluconazole induces formation of pores in yeast cells to evacuate their cytoplasmic contents (Fig. 6b, c, f). Many cell walls became incomplete or detached and some were broken. Their contents were either disappeared or were denatured (Fig. 6e, b). The cell shapes became irregular, and the organelles atrophied (Fig. 6g) and the organelles were completely disappeared (Fig. 6e, g).

In this study, fluconazole showed positive result in anticandidal activity test against *C. catenulata*, including a damaged cell wall. The results indicated that fluconazole interrupted the *Candida* cell membrane and increased the cell membrane permeability. The control *Candida* cells had uniform central density and intact cell wall (Fig. 6a, b). However, treatment with fluconazole exhibited notable alterations in the cells especially in the cell membrane and the cell wall (Fig. 6a-h). The treatment also caused the disposition of membranous bodies within the cell in addition to the decrease in the cytoplasmic volume (Fig. 6a, d). Moreover, certain number of cells apparently underwent drastic shape changes, some lysed and others collapsed (Fig. 6e). It seems that fluconazole induced dysfunctions of the cell membrane and the cell lost its metabolic functions.

Fig. 5 TEM micrograph of control *C. catenulata*. C, cytoplasm; CW, cell wall; CM, cytoplasmic membrane; N, nucleus; M, mitochondria. Magnifications **a** 120,000×, **b, d** 100,000×, and **c, e** 60,000×
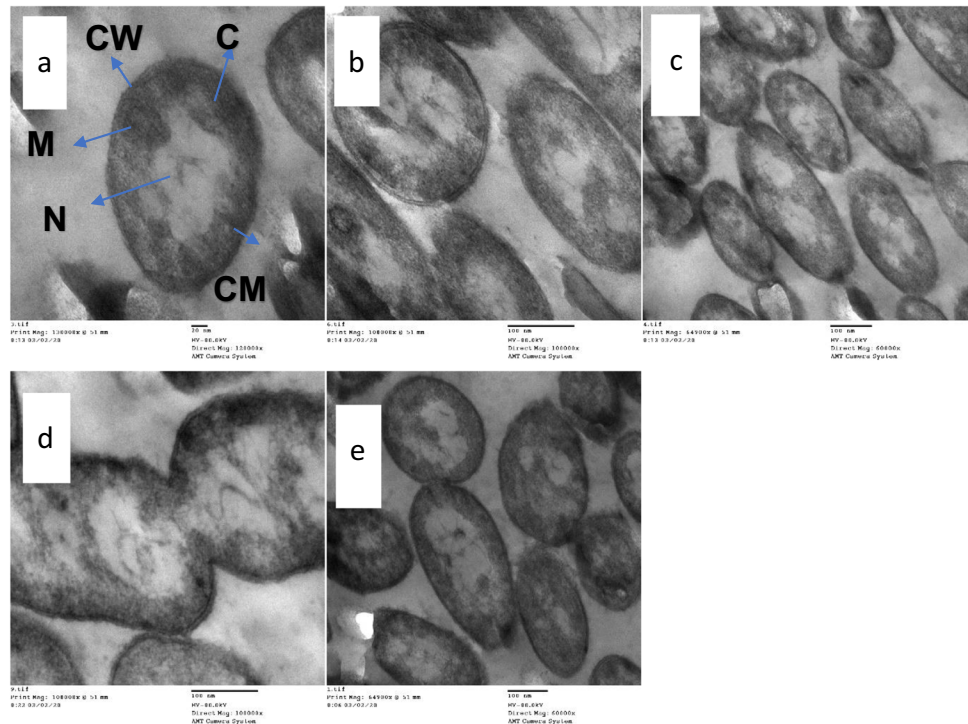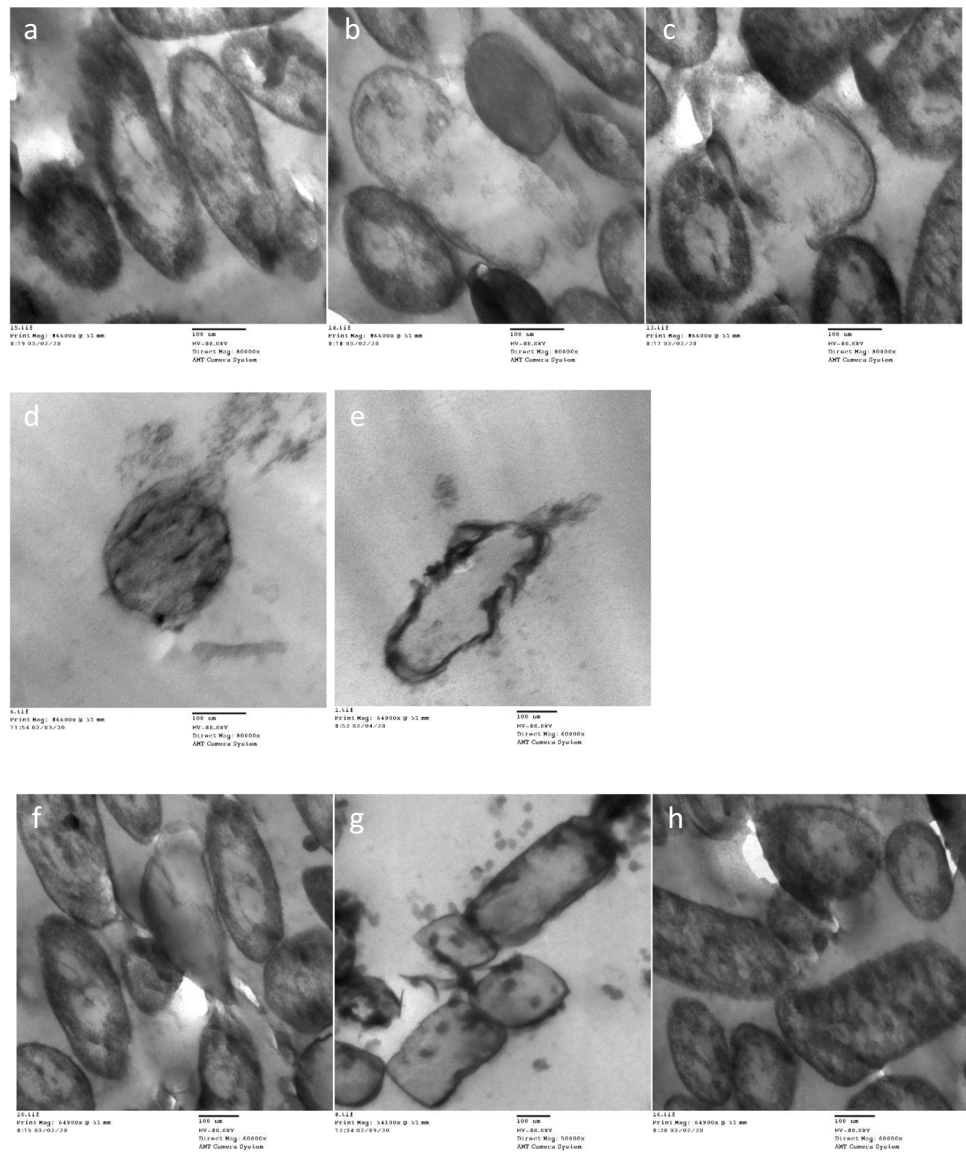
Fig. 6 TEM micrograph of fluconazole-treated cells of *C. catenulata.* Magnifications **a-d** 80,000×, **e**, **f**, **h** 60,000×, and **g** 50,000×

In the present study, fluconazole concentrations expressed destructive effects on the ultrastructure of *C. catenulata*. These deformations may be attributed to its antifungal activity. Fluconazole exerts their fungistatic effect on *Candida* spp. by inhibiting their cell growths through the disruption of sterol biosynthesis and increased cell size due to an apparently high porosity level of the cell wall. In consequence, these cells disintegrated, perhaps due to increased turgor pressure [65]. Fluconazole contains a free nitrogen atom which binds 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. This inhibition prevents the conversion of lanosterol to ergosterol [84]. Ergosterol is an essential component of the fungal cytoplasmic membrane, and subsequent accumulation of 14α-methyl sterols [61]. It also serves as a bioregulator

of membrane fluidity, asymmetry, and consequently of membrane integrity in fungal cells [51], so the inhibition of 14 α-demethylase prevents the demethylation of lanosterol causing its accumulation and blocking the ergosterol production. This action causes the formation of a plasma membrane with altered structure and function [72].

In this connection, few investigators also detected those changes in the ultrastructure of some *Candida* spp. after their exposure to fluconazole concentrations. Giordani et al. [32] studied the impact of the antifungal agent fluconazole on the ultrastructure of *C. albicans*. They realized that its low concentrations (2 μg/ml) modified the cell wall structure and accumulated polysaccharides between cell wall and plasmalemma, while doubling its concentrations thickened the cell wall with no appearance of multilayer structure and formation of electron-dense lamella and increased the accumulation of the polysaccharides. In addition, Sohnle et al.

[73] mentioned that exposing the cells of *C. albicans* to 1 µg/ml fluconazole has direct fungicidal activity on non-proliferating yeast cells by damaging the cell wall, cell membrane complex, and the interior contents of the cells.

4 Conclusion and future perspective

Maximum lipase production and biomass gain of *C. catenulata* could be obtained in medium composed of 150 mg fluconazole, 2.5% Tween 80, 0.7% yeast extract, and 10 mM $CuSO_4.5H_2O$, pH 5.5 at 37 °C with dark agitation, after 3-day incubation. This study concluded that the cultural condition plays an important role in lipase production and activity of *C. catenulata*, so it must be optimized before any experiment.

Lipase, specific activity, and protein concentrations were slightly enhanced on optimum medium enriched with both fluconazole and combined mutagen [0.1 µg/ml EtBr + 0.5 mM SA + 10% EtOH], so we can conclude that fluconazole can be the key factor for increasing pathogenicity and the lipase activity for industrial purposes but if the target was to reduce the fungal pathogenicity, only the combined mutagen 0.1 µg/ml EtBr + 0.5 mM SA + 10% EtOH will be effective.

Declarations

Ethics approval and consent to participate Not applicable.

Consent for publication Not applicable.

Competing interests The authors declare no competing interests.

References

1. Akoh CC, Lee G, Shaw J (2004) Protein engineering and applications of *Candida rugosa* lipase isoforms. Lipids 39(6):513–526

2. Al-Ziadi RE (2017) The effect of mutagens agent ethidium bromide on some therapeutic properties for local Iraqi isolate Saccharomyces boulardii. Sci J Med Res 1(1):13–18

3. Alabdalall AH, Al-Anazi NA, Aldakheel LA et al (2021) Application and characterization of crude fungal lipases used to degrade fat and oil wastes. Sci Rep 11:19670

4. Alami NH, Nasihah L, Umar RLA, Kuswytasari ND, Zulaika E, Shovitri M (2017) AIP conference proceedings 1854, 020001 (2017). https://doi.org/10.1063/1.4985392

5. Ali S, Rafi H, Haq I-u (2010) Production of an extracellular lipase from Candida lipolytica and parameter significance analysis by Plackett-Burman design. Eng Life Sci 10(5):465–473

6. Amesz WJC, van der Zeijst BAM (1972) Azide as inhibitor of protein synthesis in yeast protoplasts. FEBS Lett 26(1–2):165–168

7. Bandara HMHN, Lam OLT, Jin LJ, Samaranayake L (2012) Microbial chemical signaling: a current perspective. Crit Rev Microbiol 38(3):217–249

8. Bapiraju KVVSN, Sujatha P, Ellaiah P, Ramana T (2004) Mutation induced enhanced biosynthesis of lipase. Afr J Biotechnol 3(11):618–621

9. Basma AA, Zuraini Z, Sasidharan S (2011) A transmission electron microscopy study of the diversity of Candida albicans cells induced by Euphorbia hirta L. leaf extract in vitro. Asian Pac J Trop Biomed 1(1):20–22

10. Bindiya PA, Ramana T (2014) Optimization of lipase production from an indigenously isolated marine Aspergillus sydowii of Bay of Bengal. J Biochem Biotechnol 3:198–202

11. Boonchaidung T, Papone T, Leesing R (2013) Effect of carbon and nitrogen sources on lipase production by isolated lipase-producing soil yeast. J Life Sci Technol 1(3):176–179

12. Bramono K, Yamazaki M, Tsuboi R, Ogawa H (2006) Comparison of proteinase, lipase, alpha-glucosidase activities from the clinical isolates of *Candida* species. Jpn J Infect Dis 59(2):73–76

13. Brown TA (ed) (2002) Mutation, repair and recombination, Chapter 14. In: Genomes, 2nd edn. Weley-Liss, Oxford

14. Cesário LM, Pires GP, Pereira RFS et al (2021) Optimization of lipase production using fungal isolates from oily residues. BMC Biotechnol 21:65

15. Chauhan NM, Raut JS, Karuppayil SM (2011) A morphogenetic regulatory role for ethyl alcohol in *Candida albicans*. Mycoses 54(6):e697–e703

16. Chi Z, Zhe Chi, Zhang T, Liu G, Li J, dan Wang X (2009) Production, characterization and gene cloning of the extracelullar enzymes from the marine-derived yeasts and their potential applications. Biotechnol Adv 27:236–255

17. Cihangir N, Sarikaya E (2004) Investigation of lipase production by a new isolate of Aspergillus sp. World J Microbiol Biotechnol 20:193–197

18. Colla LM, Primaz AL, Benedetti S, Loss RA, de Lima M, Reinehr CO, Bertolin TE, Costa JA (2016) Surface response methodology for the optimization of lipase production under submerged fermentation by filamentous fungi. Braz J Microbiol 47(2):461–467

19. Csutak O, Sabau E, Pelinescu D, Corbu V, Cirpici I, Vassu T (2016) Fatty acids effect on lipase and biosurfactant induction in *Rhodotorula Glutinis* CMGB-RG5. AgroLife Sci J 5(1):51–58

20. Deive FJ, Costas M, Longo MA (2003) Production of a thermostable extracellular lipase by *Kluyveromyces marxianus*. Biotechnol Lett 25:1403–1406

21. Demir H, Tari C (2015) Effect of physicochemical parameters on the polygalacturonase of an *Aspergillus sojae* mutant using wheat bran, an agro-industrial waste, via solid state fermentation. J Sci Food Agric 96(10):3575–3582

22. Dimitris P, Paul C, Dimitris K, Basil JM (1992) Optimizing production of extracellular lipase from *Rhodotorula* glutinis. Biotechnol Lett 14:397–402

23. Dinauer MC, MacKay SA, Devreotes PN (1980) Cyclic 3, 5-AMP relay in *Dictyostelium discoideum* III. The relationship of cAMP synthesis and secretion during the cAMP signaling response. J Cell Biol 86:537–545

24. Dornelas-Ribeiro M, Pinheiro EO, Guerra C, Braga-Silva LA, Carvalho SMFD, Santos ALSD, Rozental S, Fracalanzza SEL (2012) Cellular characterisation of *Candida tropicalis* presenting fluconazole-related trailing growth. Mem Inst Oswaldo Cruz 107(1):31–38

25. Doukyu N, Ogino H (2010) Organic solvent-tolerant enzymes. Biochem Eng J 48:270–282

26. El-Batal AI, Farrag AA, Elsayed MA, El-Khawaga AM (2016) Effect of environmental and nutritional parameters on the extracellular lipase production by *Aspergillus niger*. Int Lett Nat Sci 60:18–29

27. El-Sawah MM, Sherief AA, Bayoumy SM (1995) Enzymatic properties of lipase and characteristics production by *Lactobacillus delbrueckii subsp bulgaricus*. Antonic van Leeuwenhock 67(4):357–362

28. Elegado F, Legaspi CL, Paet JM, Querubin F, Tolentino JE, Vilela J, JrA Paguio, Maloles J, Zarate J (2019) Screening, identification and optimization of extracellular lipase production of yeast (*Cryptococcus flavescens*) isolated from a tree canopy fern in the Mount Makiling Forest Reserve, Philippines. AIP Conf Proc 2155:020029. https://doi.org/10.1063/1.5125533

29. Ezema BO, Kingsley O, Omeje RM, Bill AD, Goddard SO, Eze O, Fernandez-Castane A (2022) Bioinformatic characterization of a triacylglycerol lipase produced by *Aspergillus flavus* isolated from the decaying seed of *Cucumeropsis mannii*. J Biomol Struct Dyn. https://doi.org/10.1080/07391102.2022.2035821

30. Gaspar ML, Cunningham M, Pollero R, Cabello M (1999) Occurrence and properties of an extracellular lipase in *Mortierella vinacea*. Mycologia 91:108–113

31. Ghannoum MA, Rice LB (1999) Antifungal agents: mode of action, mechanisms of resistance, and correlation of these mechanisms with bacterial resistance. Clin Microbiol Rev 12(4):501–517

32. Giordani R, Gachon C, Moulin-Traffort J, Regli P (1997) A synergistic effect of Carica papaya latex sap and fluconazole on *Candida albicans* growth. Mycoses 40(11–12):429–437

33. Gupta R, Gupta N, Rathi P (2004) Bacterial lipases: an overview of production, purification and biochemical properties. Appl Microbiol Biotechnol 64(6):763–781

34. Ha KC, White TC (1999) Effects of azole antifungal drugs on the transition from yeast cells to hyphae in susceptible and resistant isolates of the pathogenic yeast *Candida albicans*. Antimicrob Agents Chemother 43(4):763–768

35. Hafez RM, Abdel-Rahman TMA, Abed NN, Mostafa EE, Atef A (2020) Purification and characterization of the virulent factor lipase extracted from *Candida catenulata*. Plant Arch 20(2):7069–7079

36. Hasan F, Shah AA, Hameed A (2006) Industrial applications of microbial lipases. Enzyme Microb Technol 39(2):235–251

37. Hornby JM, Nickerson KW (2004) Enhanced production of farnesol by *Candida albicans* treated with four azoles. Antimicrob Agents Chemother 48(6):2305–2307

38. Jatta B, Gunasekaran M, Mohan N (2009) Influence of cultural conditions on lipase production in *Candida albicans*. Asian J Biotechnol 1(3):118–123

39. Jayaraman R, Ilyas MM (2010) Strain improvement of pseudomonas sp for the production of lipase. J Exp Sci 1(11):1–3

40. Joseph B, Ramteke PW, Kumar PA (2006) Studies on the enhanced production of extracellular lipase by *Staphylococcus epidermidis*. J Gen Appl Microbiol 52:315–320

41. Joshie KR, Wheeler EE, Gavin JB (1975) The ultrastructure of *Candida krusei*. Mycopathologia 56:5–8

42. Kamini N, Fujii T, Kurosu T, Iefuji H (2000) Production, purification and characterization of an extracellular lipase from the yeast, Cryptococcus sp S-2. Process Biochem 36(4):317–324

43. Kanasawud P, Phutrakul S, Bloomer S, Aldercrentz P, Mattiassen B (1992) Triglyceride interesterification by lipases.3. Alcoholysis of pure triglycerides. Enzyme Microb Technol 14:959–964

44. Karanam SK, Medicherla NR (2008) Enhanced lipase production by mutation induced *Aspergillus japonicas*. Afr J Biotechnol 7(12):2064–2067

45. Kleinhofs A, Owais WM, Nilan RA (1978) Azide. Mutat Res 55(3–4):165–195

46. Kotogán A, Németh B, Vágvölgyi C, Papp T, Takó M (2014) Screening for extracellular lipase enzymes with transesterification capacity in Mucoromycotina strains. Food Technol Biotechnol 52:73–82

47. Leblond-Larouche L, Morais R, Zollinger M (1979) Studies of the effect of chloramphenicol, ethidium bromide and camptothecin on the reproduction of *Rous sarcoma* virus in infected chick embryo cells. J Gen Virol 44:323–331

48. Li N, Zong MH (2010) Lipases from the genus *Penicillium*: production, purification, characterization and applications. J Mol Catal B: Enzym 66(1–2):43–54

49. Lin ES, Wang CC, Sung SC (2006) Cultivating conditions influence lipase production by the edible Basidiomycete *Antrodia cinnamomea* in submerged culture. Enzyme Microb Technol 39(1):98–102

50. Lowry O, Roseborough N, Farr A, Randall R (1951) Protein measurement with folin phenol reagent. J Biol Chem 193(1):265–275

51. Lv QZ, Yan L, Jiang YY (2016) The synthesis, regulation, and functions of sterols in *Candida albicans*: well-known but still lots to learn. Virulence 7(6):649–659

52. Mahanta N, Gupta A, Khare SK (2008) Production of protease and lipase by solvent tolerant *Pseudomonas aeruginosa PseA* in solid-state fermentation using *Jatropha curcas* seed cake as substrate. Bioresour Technol 99(6):1729–1735

53. Maliszewska I, Mastalerz P (1992) Enzyme Microb Technol 14:190–193

54. Mensah JK, Obadoni BO, Akomeah PA, Ikhajiagbe B, Ajibolu J (2006) The effects of sodium azide and colchicine treatments on morphological and yield traits of sesame seed (*Sesame indicum* L.). Afr J Biotechnol 6(5):534–538

55. Messias JM, da Costa BZ, de Lima VM, Dekker RF, Rezende MI, Krieger N, Barbosa AM (2009) Screening *Botryosphaeria* species for lipases: production of lipase by *Botryosphaeria ribis* EC-01 grown on soybean oil and other carbon sources. Enzym Microbiol Technol 45(6–7):426–431

56. Muñoz JA, Rossi D, Ishida K, Spadari CC, Melhem M, Garcia DM, Caires A, Taborda CP, Rodrigues EG (2017) Antifungal activity of the biphosphinic cyclopalladate C7a against *Candida albicans* yeast forms in vitro and in vivo. Front Microbiol 8:771

57. Obe G, Ristow H (1979) Mutagenic, cancerogenic and teratogenic effects of alcohol. Mutat Res 65(4):229–259

58. Owais WM, Kleinhofs A (1988) Metabolic activation of the mutagen azide in biological systems. Mutat Res-Fund Mol M 197(2):313–323

59. Pappas PG, Kauffman CA, Andes DR, Clancy CJ, Marr KA, Ostrosky-Zeichner L, Reboli AC, Schuster MG, Vazquez JA, Walsh TJ, Zaoutis TE (2016) Clinical practice guideline for the management of candidiasis: 2016 update by the infectious diseases society of America. Clin Infect Dis 62(4):e1–e50 (**10**)

60. Payá-Tormo L, Rodríguez-Salarichs J, Prieto A, Martínez MJ, Barriuso J (2019) Improvement of the activity of a fungal versatile-lipase toward triglycerides: an in silico mechanistic description. Front Bioeng Biotechnol 7:71

61. Pfizer Australia Pty Ltd (2004) Diflucan (Australian Approved Product Information). Pfizer Australia, West Ryde

62. Radha S, Babu RH, Sridevi A (2012) Development of mutant fungal strains of *Aspergillus niger* for enhanced production of acid protease in submerged and solid-state fermentation. Eur J Exp Biol 2(5):1517–1528

63. Radman M (1999) Enzymes of evolutionary change. Nature 401:866–868

64. Rajan A, Nair AJ (2011) A comparative study on alkaline lipase production by a newly isolated *Aspergillus fumigatus* MTCC 9657 in submerged and solid-state fermentation using economically and industrially feasible substrate. Turk J Biol 35(5):569–574

65. Ramani R, Chaturvedi V (2000) Flow cytometry antifungal susceptibility testing of pathogenic yeasts other than *Candida albicans* and comparison with the NCCLS broth microdilution test. Antimicrob Agents Chemother 44:2752–2758

66. Rehman S, Bhatti HN, Bhatti IA, Asgher M (2011) Optimization of process parameters for enhanced production of lipase by *Penicillium notatum* using agricultural wastes. Afr J Biotechnol 10(84):19580

67. Reynolds ES (1963) The use of lead citrate at high pH as an electron opaque stain in electron microscopy. J Cell Biol 17:208–212

68. Salihu A, Alam MdZ, Abdulkarim M, Salleh HM (2011) Optimization of lipase production by *Candida cylindracea* in palm oil mill effluent based medium using statistical experimental design. J Mol Catal B Enzym 69(1–2):66–73

69. Salwoom L, Raja Abd Rahman R, Salleh AB, Mohd Shariff F, Convey P, Pearce D, Mohamad Ali MS (2019) Isolation, characterisation, and lipase production of a cold-adapted bacterial strain *Pseudomonas* sp. LSK25 Isolated from Signy Island, Antarctica. Molecules (Basel, Switzerland) 24(4):715

70. Sarker S, Sreekanth B, Kant S, Banerjee R, Bhattacharyya BC (1998) Production and optimization of microbial lipase. Bioprocess Eng 19:29–32

71. Sharma D, Sharma B, Shukla AK (2011) Biotechnological approach of microbial lipase: a review. Biotechnology 10(1):23–40

72. Sheehan DJ, Hitchcock CA, Sibley CM (1999) Current and emerging azole antifungal agents. Clin Microbiol Rev 12(1):40–79

73. Sohnle PG, Hahn BL, Fassel TA, Kushnaryov VM (1998) Analysis of fluconazole effect on Candida albicans viability during extended incubations. Med Mycol 36(1):29–36

74. Subroto E, Indiarto R, Pangawikan AD, Huda S, Yarlina VP (2020) Characteristics, immobilization, and application of Candida rugosa lipase: a review. Food Res 4(5):1391–1401

75. Szekeres A, Kredics L, Antal Z, Kevei F, Manczinger L (2004) Isolation and characterization of protease overproducing mutants of *Trichoderma harzianum*. FEMS Microbiol Lett 233(2):215–222

76. Teng Y, Xu Y (2008) Culture condition improvement for whole-cell lipase production in submerged fermentation by *Rhizopus chinensis* using statistical method. Bioresour Technol 99(9):3900–3907

77. Thakur S (2012) Lipases, its sources, properties and applications: a review. Int J Sci Eng Res 3:1–29

78. Tomchick R, Mandel HG (1964) Biochemical effects of ethidiurn bromide in micro -organisms. J Gen Microbiol 36:225–236

79. Toscano L, Gochev V, Montero G, Stoytcheva M (2011) Enhanced production of extracellular lipase by novel mutant strain of *Aspergillus Niger*. Biotechnol Biotechnol Equip 25(1):2243–2247

80. Tsuboi R, Komatsuzaki H, Ogawa H (1996) Induction of an extracellular estrase from *Candida albicans* and some of its properties. Infect Immun 64(8):2936–2940

81. Ulker S, Ozel A, Colak A, Karaoglu SA (2011) Isolation, production and characterization of an extracellular lipase from *Trichoderma harzianum* isolated from soil. Turk J Biol 35(5):543–550

82. Velmurugan P, Lee YH, Venil CK, Lakshmanaperumalsamy P, Chae JC, Oh BT (2010) Effect of light on growth, intracellular and extracellular pigment production by five pigment-producing filamentous fungi in synthetic medium. J Biosci Bioeng 109(4):346–350

83. Verma S, Meghwanshi GK, Kumar R (2021) Current perspectives for microbial lipases from extremophiles and metagenomics. Biochimie 182:23–36

84. Wu Y, Gao N, Li C, Gao J, Ying C (2017) A newly identified amino acid substitution T123I in the 14α-demethylase (Erg11p) of *Candida albicans* confers azole resistance. FEMS Yeast Res 17(3):1–6

85. Yong SK, Lim BH, Saleh S, Tey LH (2016) Optimisation, purification and characterisation of extracellular lipase from *Botryococcus sudeticus* (UTEX 2629). J Mol Catal B: Enzym 126:99–105

86. Zhang BH (2000) Regulation of plant growth regulators on cotton somatic embryogenesis and plant regeneration. Biochem 39:15–67