

Characterization of Lipases from a Vegetable Oil Contaminated Soil Fungal Isolates

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Abstract Lipases are enzymes that hydrolyse lipids to produce free fatty acids and glycerol. Fungi were cultured on sabouraud dextrose agar (SDA) plates and identified using microscopic techniques. Screening for lipase producers was carried out on SDA media supplemented with 3 % olive oil at ambient temperature. *M. pusillus*, *M. canis*, *A. fumigatus*, *Yeast* and *T. mentagrophytes* were found to produce lipases in different amounts with *Yeast* and *M. canis* being the highest producers, they were further characterised for this reason. Lipase production was carried out using submerged fermentation. Both *Yeast* and *M. canis* produced lipases maximally at 72 h. Optimum pH and temperature of activity for the lipases from *Yeast* were determined to be 5 and 35 °C respectively whereas, those from *M. canis* were 6 and 40 °C correspondingly. *Yeast* and *M. canis* lipases had preference for olive oil than vegetable and palm oils and both enzymes were activated by K⁺, Mg²⁺ and Ca²⁺ and inhibited by Fe³⁺, Hg²⁺ and Co²⁺. The lipase enzyme from *Yeast* had V_{max} of 0.0006 U/mL/Sec, K_m of 0.4242 mM and K_{cat} of 0.0004 S⁻¹ while that from *M. canis* had corresponding V_{max}, K_m and K_{cat} of 0.0001 U/mL/Sec, 3.2287 mM and 0.0001 S⁻¹.

Keywords Fungal Isolates, *Yeast*, *Microsporum canis*, Lipases, Characterization

1. Introduction

Lipases (E.C. 3.1.1.3) are biological catalysts that break down fats and oils releasing free fatty acids and glycerol. Additionally, they participate in esterification and transesterification reactions, lactonization, aminolysis [1], acidolysis [2], aminolysis, alcoholysis, hydrolysis [3], [4] and interesterification reactions in non-aqueous media [5]. These applications are however due to the potentials of lipases to catalyze both synthetic and hydrolytic processes. Worthy of note also, is their possession of distinctive properties such as, non-requirement of cofactors [2], wide spectrum of substrate specificities, stereoselectivity, chemoselectivity, regioselectivity, stability in organic solvents and their ability to catalyze varied reactions in both water soluble and non-water soluble systems [3]. Lipases of microbial origin differ based on the strain of the organism, composition of the growth medium, sources of nitrogen, temperature, carbon and pH [6], [7].

The all-round properties of lipases make them to have many potential applications in diverse aspects including, food processing, detergent, pharmaceutical, cosmetics, textile, agrochemical [6], paper, dairy, oleo-chemicals and leather industries [2]. They also find usefulness in the management of waste water, fine chemicals synthesis [6] and the production of other important bioactive compounds such as polyunsaturated fatty acids and carotenoids [8]. Fungi mostly produce lipases extracellularly thereby

making their extraction easy. Fungi and other microorganisms that produce lipases are present in different habitats such as, crude and vegetable oil contaminated soils, decaying foods, oil seeds [2], fish intestine [6].

Soils particularly the ones contaminated with vegetable oil have been reported to contain lipolytic enzymes which often degrade the fats and oils in them [9]. Filamentous fungi of the many species of genera *Mucor*, *Candida* [10], *Aspergillus*, *Rhizopus*, *penicillium* and *trichoderma* have been reported to be the most prospective lipase producers [11] but despite this, many microorganisms having the ability to produce lipases which are able to breakdown fats and oils are yet to be isolated, identified and characterized [6]. [25] reported that only a very small number (about 2%) of microorganisms have been used as sources of enzyme in the whole wide world which have served mankind for the different products they are used to produce. Submerged fermentation is preferred to solid state fermentation because of the fact that growth conditions are easily controlled but the cost of substrates for enzyme production still remains a problem for any successful enzyme application presenting about 10 – 30 % of the overall costs [1]. Therefore, in the present investigation, lipases production was carried out using submerged fermentation and olive oil as a substrate; production and characterization of lipases from two fungal strains were carried out with possible characteristics for desired applications.

2. Materials and Methods

2.1. Sample Collection

Soil samples were collected into sterile plastic bags from the dump site of Grand Cereals Limited Factory, Plateau State, Nigeria (known mainly for the production of Grand Pure Soya Oil) using a sterile spatula after which they were placed immediately in a flask packed with ice and brought to the laboratory. The samples were sealed in sterile containers and stored in the refrigerator at 4°C until required for further usage.

2.2. Isolation and Identification of Lipolytic Fungal Species from Vegetable Oil Contaminated Soil

A six-fold serial dilution of the soil samples was made in sterile distilled water followed by the addition of 1 mL inoculum of 10⁴ dilution on sabouraud dextrose agar (SDA) media. The fungal inoculated plates were incubated at 25 °C for 5 days. Colonies which appeared were subcultured in order to have a pure culture of each isolate and stored in SDA slants at 4 °C. Identification of the isolates was carried out by observing their growth features (such as pigmentation, texture and form) and microscopically based on the growth from their aerial

hyphae and structures like conidia, spores, hyphae and sporangiophore or conidiophore.

2.3. Preliminary Screening for Lipase Producers

The fungal isolates were screened by their ability to produce lipase following incubation on SDA media supplemented separately with 3 % (v/v) olive oil and analyzing the formation of clear zones around colonies according to the method described by [7]. The abilities of the isolates to produce lipase were at that moment compared by measuring the zone of clearance. Isolates having lipolytic halo radius (R) / colony radius (r) ratio of greater than 5 mm were selected for further studies.

2.4. Production of Lipase from Selected Fungal Strains of Vegetable Oil Contaminated Soil

Lipase production was carried out in broth containing (g/500 mL): MgSO₄ · 7H₂O (0.1), KH₂PO₄ (0.5), K₂HPO₄ (1.5), Na₂SO₄ (1.0), yeast extract (2.5), peptone (2.5), glucose (1.0) and olive oil (2% v/v), pH 7.0. Productions were carried out in 50 mL production media with inoculation of a 6 mm of the fungal spores inside a 250 mL Erlenmeyer flasks under shaking speed of 150 rpm at room temperature for successively 7 days. The broth was harvested at 24 h interval and centrifuged at 4000 rpm for 40 min at 10 °C to remove cells. Total protein as well as lipase activity were determined from the supernatant.

2.5. Assay for Lipase Activity

Lipase activity was assayed spectrophotometrically at 450 nm according to the method of [12] with slight modification. To 800 µL of substrate solution (containing 3% v/v Tween 80 in 20 mM Tris-HCl with pH of 8.5 and 80 mM CaCl₂), 200 µL of the enzyme was added. The hydrolysis rate of Tween 80 was monitored directly by measuring the change in absorbance over a period 5 min.

2.6. Total Protein Determination

Protein concentration was measured spectrophotometrically at 590 nm as described by [13]. 1 mL of the sample was added to 5 mL of Bradford reagent, the mixture was allowed to stand for 5 min at room temperature after which absorbance was read. Total protein was determined by extrapolating from the egg albumin (EA) standard curve which was constructed using a solution containing 1 mg/mL EA.

2.7. Biochemical Characterization of Lipases from Fungal Isolates of Vegetable Oil Contaminated Soil

2.7.1. Effect of pH

The effect of pH on the activity of lipases was studied by

incubating 200 μL of the enzymes with 1M Tris-HCl buffer over a pH range of 4 to 10 and assayed for lipase activity. A plot of lipase activity against pH was used to determine the optimum pH for the lipases.

2.7.2. Effect of Temperature

The effect of temperature on the activity of lipases was studied by assaying lipase activity followed by incubation at temperature range of 20 to 55 $^{\circ}\text{C}$. A plot of lipase activity against temperature was used to determine the optimum temperature of activity for the lipases.

2.7.3. Effect of Different Substrates on Lipase Activity

The effect of different substrates on lipase activities was studied; 1% Tween 80 was substituted with olive oil, palm oil and groundnut oil and assayed as described above.

2.7.4. Effect of Different Metal Ions on Lipase Activity

The effect of different metal ions on lipase activities was studied by using K^+ , Co^{2+} , Mg^{2+} , Hg^{2+} , Cu^{2+} , Ca^{2+} and Fe^{3+} . 0.2 μL of 1mM of each metal ion already prepared in 1M Tris-HCl buffer was added to the substrate mixture and treated as described in assay for lipase activity above.

2.7.5. Determination of K_m , V_{max} and K_{cat}

The kinetic constants K_m and V_{max} for lipases were determined from the Lineweaver – Burk plot following a change in substrate concentration over the range of 0.02 – 1.80 v/v of Tween 80 and assayed. K_{cat} of the lipases were as well determined.

2.7.6. Thermal Inactivation Studies

The thermal inactivation studies was carried out in a

water bath over temperature range of 20 - 90 $^{\circ}\text{C}$ using 1M Tris-HCl buffer for 30 min. Thereafter, the test tubes were allowed to cool to room temperature and assayed for lipase activity. Arrhenius plot was used to determine the energy of deactivation for the enzymes as thermal inactivation usually follows the first order kinetics [14]. The residual activity of the enzymes was also calculated in order to determine the half-life of the enzymes.

2.8. Statistical Analysis

Experiments were performed in triplicates and the means of results determined were calculated using Statistical Package for Social Science (SPSS) version 23.

3. Results and Discussion

Fungi are good sources of enzymes and reports from previous literatures have it that soils contaminated with oil often retain microbial diversity [15]. Out of the 9 fungi isolated from the vegetable oil contaminated soil following incubation on SDA, 5 isolates exhibited different magnitudes of zones of clearance when 3 % olive was used as a substrate (Table 2). This indicates the occurrence of degradation reaction of olive oil by extracellular enzymes, inducing the production of the lipase enzyme. From the 5 lipase producers, *Yeast* and *Microsporium canis* being the best with zones of hydrolysis of 5.63 and 5.16 mm respectively were selected for lipase production and characterization. Various microorganisms such as bacteria, fungi and yeast are recognised as potential producers of enzymes which employ these enzymes in nutrients degradation process [6].



Plate I

Plate II

Plate III



Plate IV

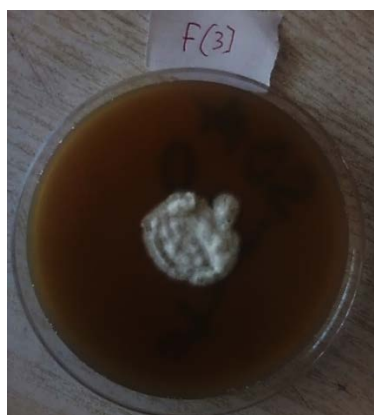


Plate V

Plates I – V: Morphological features of fungal isolates from a vegetable oil contaminated soil;
 I – *Mucor pusillus*,
 II – *Microsporium canis*,
 III – *Aspergillus fumigatus*,
 IV – *Yeast*,
 V - *Trichophyton mentagrophyte*

Table 1. Zone of hydrolysis of fungal isolates from a vegetable oil contaminated soil

S/No	Fungal isolate	Zone of hydrolysis (mm)
1	<i>Mucor pusillus</i>	4.18 ± 0.01
2	<i>Microsporium canis</i>	5.16 ± 0.02
3	<i>Aspergillus fumigatus</i>	3.27 ± 0.02
4	<i>Yeast</i>	5.61 ± 0.02
5	<i>Trichophyton mentagrophyte</i>	1.82 ± 0.10

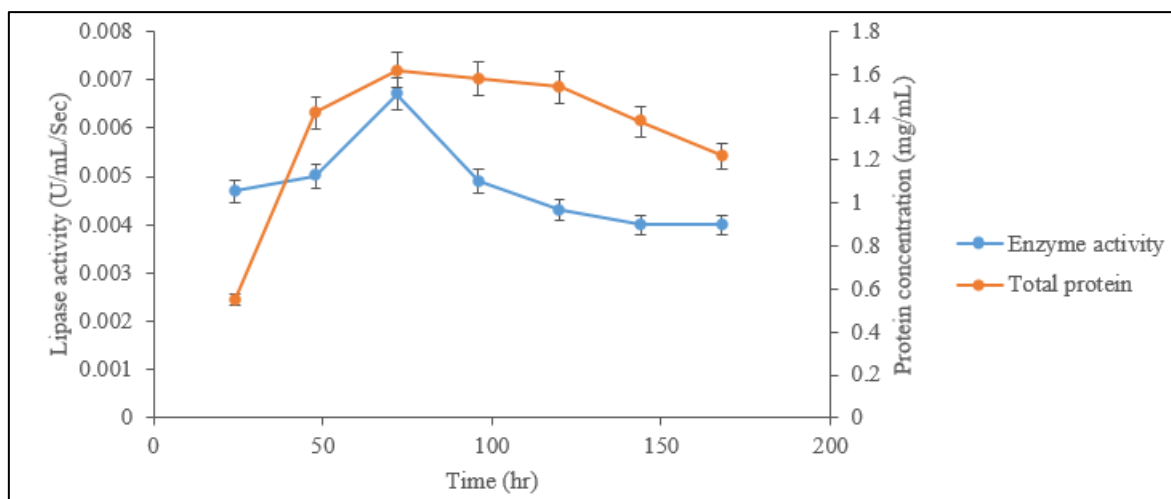


Figure 1. Effect of incubation time on lipase production by *Yeast* isolated from a vegetable oil contaminated soil

Quantitative lipase production was carried out using submerged fermentation for a period of 7 days during which lipase activity and total protein were determined. Both *Yeast* and *Microsporium canis* produced lipases maximally at 72 h (Figures 1 and 2). A number of other studies also reported lipase production by submerged fermentation. [16] reported maximum lipase production from *Candida rugosa* at 50 h using olive oil as a substrate and [17] on the other hand reported maximum lipase production using olive mill wastewater from *Candida cylindracea* NRRLY-175 at 175 h. The time of incubation for enzyme production is usually guided by the

characteristics of the culture medium and is dependent on growth rate [18].

Lipase from *Yeast* had an optimum pH of 5 while that from *M. canis* was determined to be 6 (Figure 3). Various studies with lipases reported acidic pH values which correlate with the acidic pH values of the present study. [19] reported an optimum pH of 6.5 from *A. niger* and [8] reported a pH value of 5.2 for the *Rh. oryzae* enzyme studied. Both lipases found in the present study may be useful in the leather industry because the use of acidic active lipases has been reported in the treatment of animal skins which have been kept in pickled form [8].

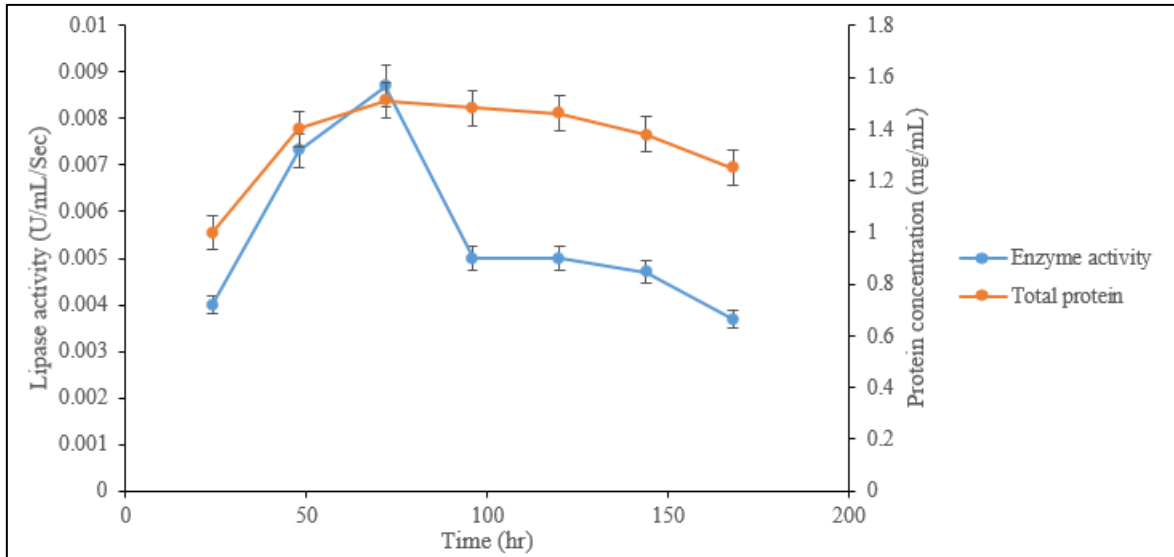


Figure 2. Effect of incubation time on lipase production *Microsporium canis* isolated from a vegetable oil contaminated soil

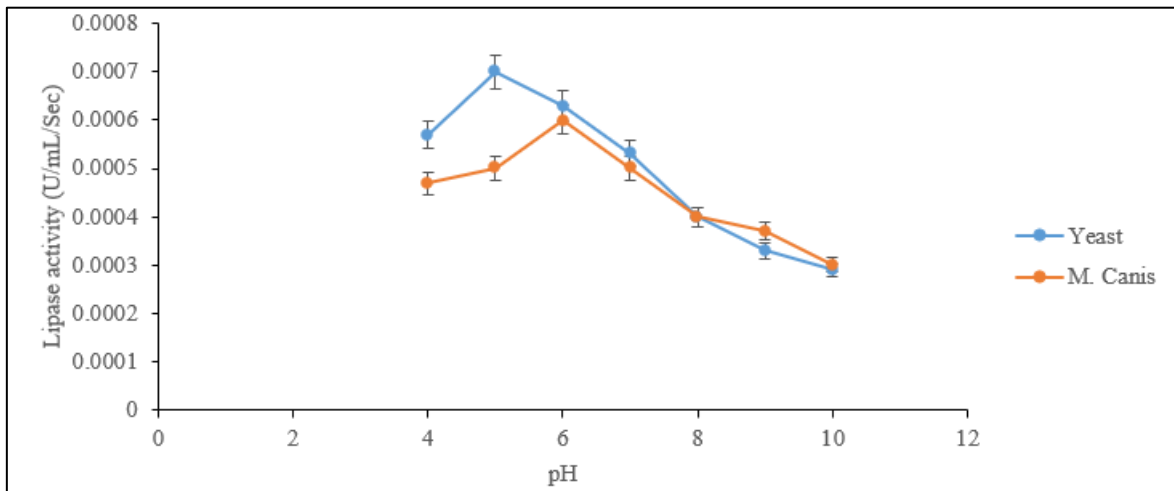


Figure 3. Effect of pH on the activity of lipases from fungal isolates of a vegetable oil contaminated soil

Figure 4 presents the optimum temperature of activity of lipases from *Yeast* and *M. canis* isolates of a vegetable oil contaminated soil. Optimum lipase activities were determined to be 35 and 40 °C for *Yeast* and *M. canis* respectively. [19] reported 30-35 °C as the optimum temperature for an olive oil-induced lipase from *Aspergillus niger* MYA 135 while [14] reported that lipase from *Cellulomonas flavigena* UNP3 was optimally active at 30 °C. The optimum temperature obtained for lipase from *Yeast* corresponds to the highest temperature as reported by [19] and that of lipase from *M. canis* tallies with the optimum temperature of 40 °C as reported by [20] and [21] from *Aneurinibacillus aneurinilyticus* strain LP-II and *A. niger* respectively. Both lipases from *Yeast* and *M. canis* had moderate temperature values making them possibly useful in bioprocesses operating at moderate temperatures.

Palm, groundnut and olive oils were substituted with

tween 80 in order to determine their effects on lipase activities (Table 2). Generally, activities were found to be highest when olive oil was used as a substrate in this study. Palm and vegetable oils had the same activities for the lipase enzyme from *Yeast* while the lipase from *M. canis* had preference for palm oil to vegetable oil. The findings of the present study conform to those of other studies [22], [23], [24] suggesting olive oil as potent substrate for lipases.

Table 2. Effect of different substrates on the activity of lipases from fungal isolates of a vegetable oil contaminated soil

Isolate	Lipase activity (U/mL/Sec)		
	Palm oil	Groundnut oil	Olive oil
<i>Yeast</i>	0.0007 ± 1.2 × 10 ⁻⁴	0.0007 ± 5.8 × 10 ⁻⁵	0.0008 ± 5.8 × 10 ⁻⁵
<i>Microsporium canis</i>	0.0005 ± 5.8 × 10 ⁻⁵	0.0004 ± 1.2 × 10 ⁻⁴	0.0006 ± 5.8 × 10 ⁻⁵

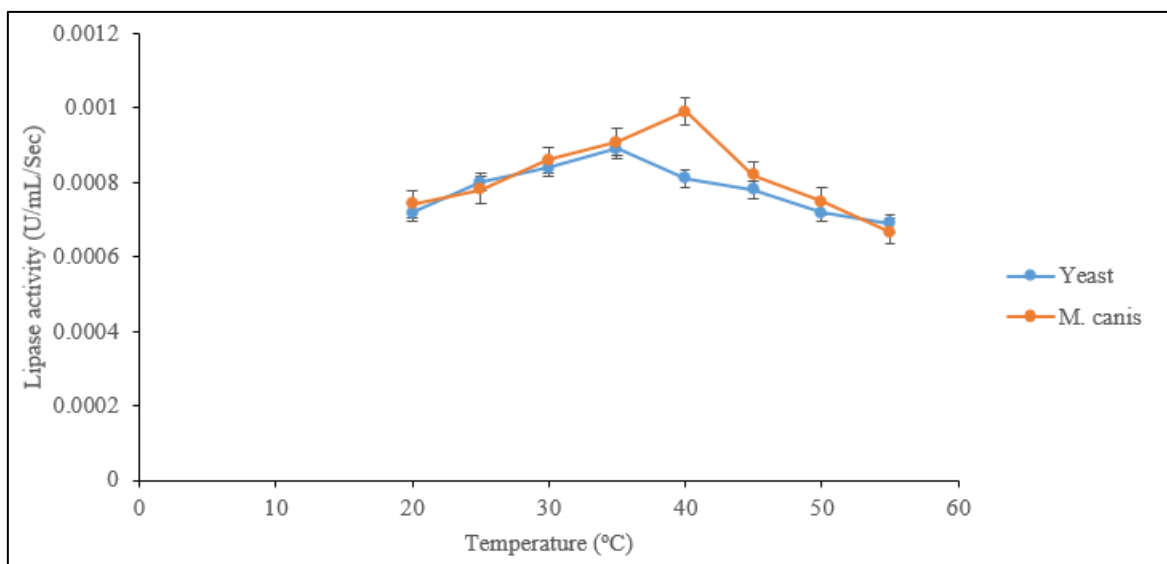


Figure 4. Effect of temperature on the activity of lipases from fungal isolates of a vegetable oil contaminated soil

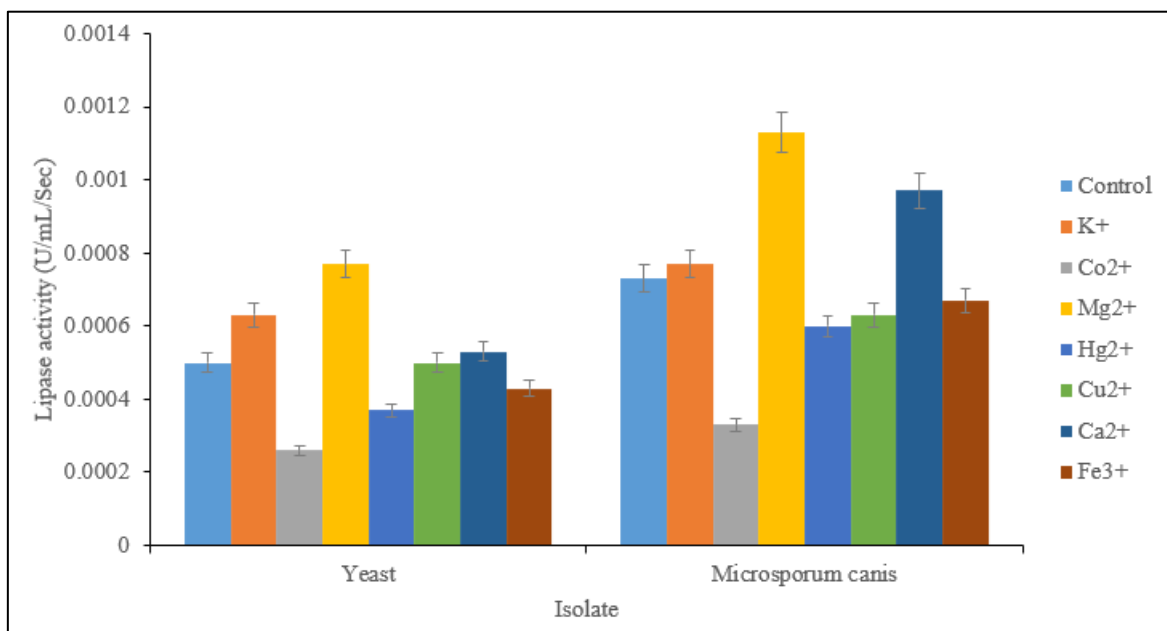


Figure 5. Effect of different metal ions on the activity of lipases from fungal isolates of a vegetable oil contaminated soil

The effect of metal ions on the activities of lipases from *Yeast* and *M. Canis* were investigated and the results are shown in Figure 5. While Cu^{2+} had no effect on lipase activity from *Yeast*, it inhibited the activity of lipase from *M. canis*. K^+ , Mg^{2+} , and Ca^{2+} increased the activity of lipases from both *Yeast* and *M. canis*. [14] reported an activation of lipase activity by Mg^{2+} and Ca^{2+} from different studies which is in tandem to the outcomes of the present study. On the other hand, Fe^{3+} , Hg^{2+} and Co^{2+} showed inhibitory effects for the lipases studied.

Figures 6 and 7 present the Lineweaver-Burk plot for the determination of K_m , V_{max} and K_{cat} from *Yeast* and *M. canis*. Corresponding V_{max} , K_m and K_{cat} values of 0.0006 U/mL/Sec, 0.4242 mM and 0.0004 S^{-1} were

determined for lipase from *Yeast* while those from *M. canis* were determined to be 0.0001 U/mL/Sec, 3.2287 mM and 0.0001 S^{-1} respectively. Other studies with lipases reported higher K_m values compared to the result of lipase from *Yeast*; [1] determined a K_m value of 9.93 mM for lipase from *Penicillium sp.* using *p*-Nitrophenyl palmitate as a substrate and [8] found an estimated value of 1.13 and 1.08 mM for purified lipases corresponding to *Rhizomucor miechei* and *Rhizopus oryzae* using *p*-nitrophenyl palmitate (*p*NPP) as substrate. It can therefore be concluded that lipases from *Yeast* has higher affinity for the substrate than the already mentioned suggesting it to be a potential biotechnological machinery.

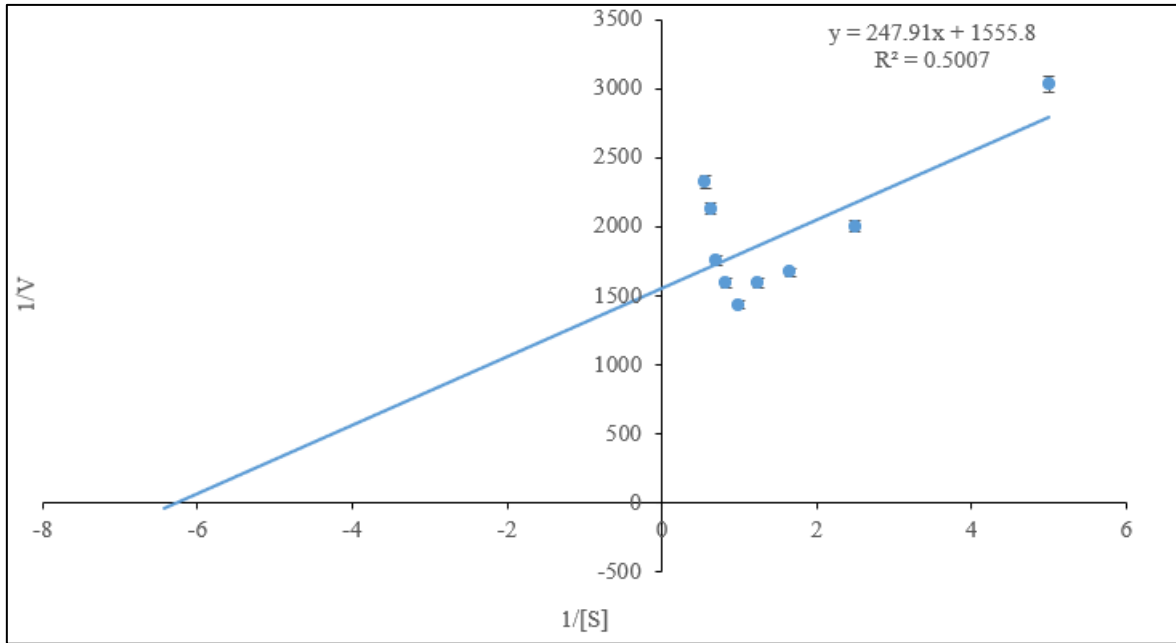


Figure 6. Lineweaver-Burk plot of lipase from *Yeast* isolated from a vegetable oil contaminated soil

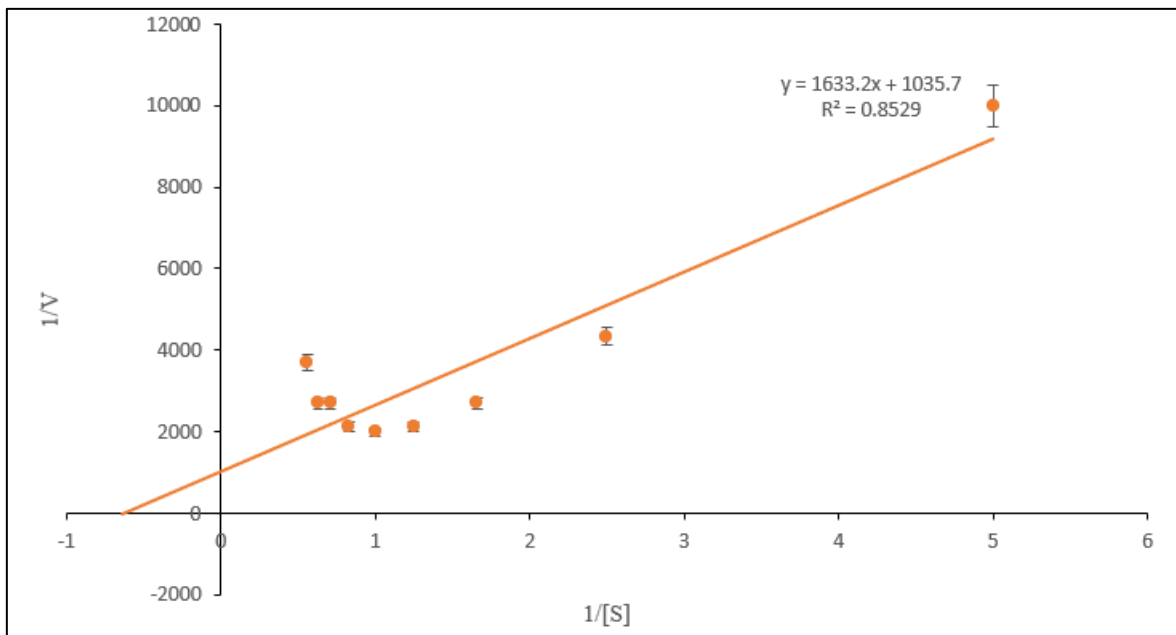


Figure 7. Lineweaver-Burk plot of lipase from *Microsporium canis* isolated from a vegetable oil contaminated soil

Lipases from *Yeast* and *M. canis* had energy of deactivation values of 22.871 and 17.923 KJ/mol respectively (Figures 8 and 9). The energy of deactivation determined in the present study were lower than the value

of 64.32 KJ/mol of lipase from *Cellulomonas flavigena* UNP3 earlier reported by [14]. The calculated half-life for lipase from *Yeast* was 0.23 hr whereas that from *M. canis* was 0.58 hr.

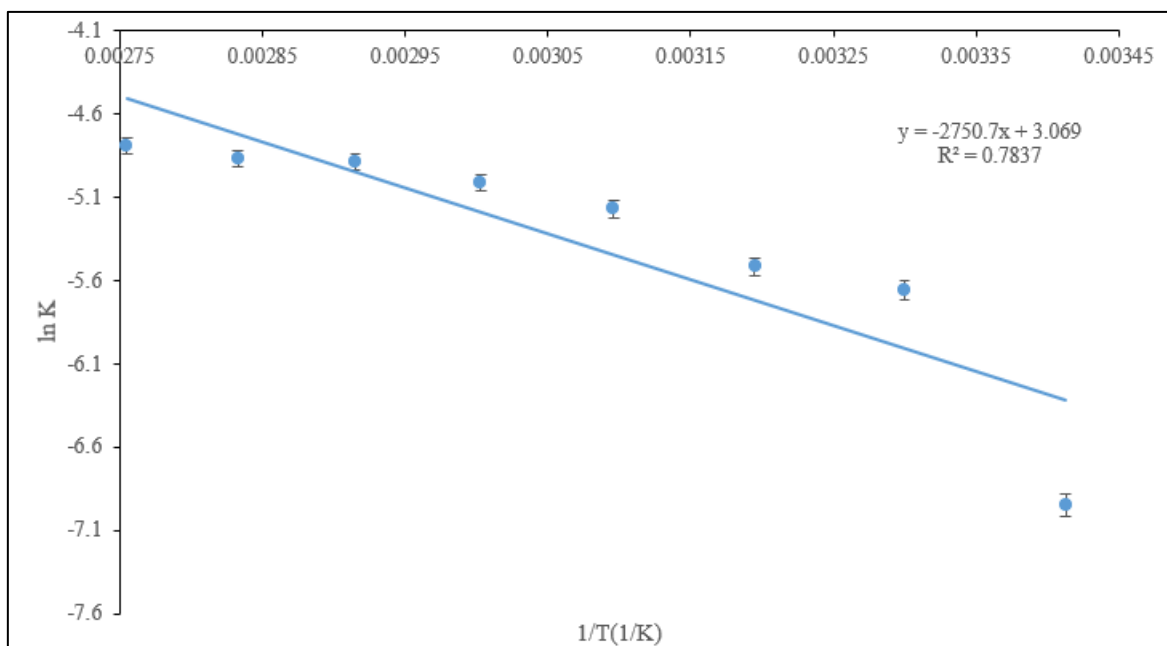


Figure 8. Arrhenius plot for determination of energy of deactivation of lipase from *Yeast* isolated from a vegetable oil contaminated soil

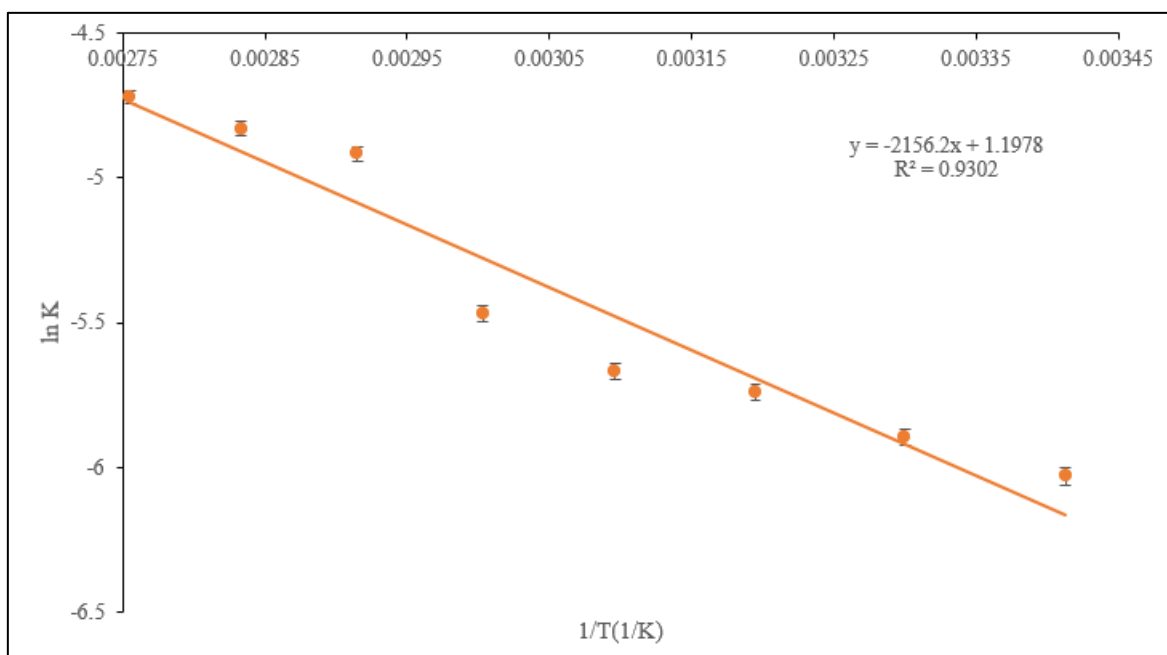


Figure 9. Arrhenius plot for determination of energy of deactivation of lipase from *Microsporium canis* isolated from a vegetable oil contaminated soil

4. Conclusions

Yeast and *M. canis* fungal strains isolated from a vegetable oil contaminated soil were found to be good lipase producers. The lipases studied were optimally active at acidic pH and at moderate temperature with immense affinities for different substrates. The findings of the present study suggest lipases from these sources could find usefulness in different bioprocesses.

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