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An Alkaline Lipase from *Scenedesmus obliquus*: Purification, Characterization and Biotechnological Potential

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Abstract: ***Algae represent an underexplored and potentially valuable source of enzymes with applications in industrial biotechnology, bioenergy, food processing, and nutraceutical production. In the present study, an intracellular lipase from the freshwater microalga Scenedesmus obliquus S6, isolated from the Waghur River near Skegaon, Jalgaon, Maharashtra, India, was purified and biochemically characterized. The enzyme was initially extracted and subjected to 80% (v/v) acetone precipitation, followed by Sephadex G-100 gel filtration chromatography. The final purification step resulted in a 6.79-fold purification with a total yield of 12.95% and a specific activity of 2.381 U/mg protein. The purified lipase exhibited an apparent molecular mass of approximately 36 kDa. Enzyme activity was detected over a temperature range of 30–60°C, with maximum activity at 40°C, while the enzyme retained activity across a broad pH range of 4.0–11.0 and showed optimum activity at pH 8.0, indicating its alkaline nature and broad pH tolerance. The enzyme was activated by Mg^{2+}, achieving 112% relative activity, and its activity was enhanced in the presence of SDS, Tween 80, Triton X-100, and EDTA, with relative activities of 120%, 108%, 104%, and 106%, respectively. In contrast, several organic solvents, metal ions, urea, and guaiacol caused varying degrees of inhibition, with Hg^{2+} and urea producing particularly pronounced inhibitory effects. Overall, the purified S. obliquus lipase exhibited notable catalytic activity, broad pH tolerance, and stability under diverse chemical conditions, highlighting its potential as a promising algal enzyme for biotechnological applications, particularly processes requiring alkaline and chemically tolerant lipases.***

Keywords: Algae; Alkaline lipase; Chromatography; Kinetic studies; Purification; Scenedesmus obliquus

1. Introduction

Lipases (triacylglycerol acyl hydrolases, *e.g.,* 3.1.1.3) are a class of enzymes that catalyze the hydrolysis of long-chain triglycerides. Microbial lipases are produced by fungal, yeast, and bacterial species. The following microorganisms account for most industrial microbial lipase production: *Candida* sp., *Aspergillus* sp., *Rhizomucor* sp., *Rhizopus* sp., *Humicola* sp., *Yarrowia lipolytica*, and *Pseudomonas* sp. [1]. Lipases are ubiquitous enzymes reported from plants, animals, and microbes. Lipases have great potential in medical applications [2]. In addition to their natural ability to hydrolyse carboxylic ester bonds, lipases can catalyze esterification, interesterification, and transesterification reactions in nonaqueous media. This versatility makes lipases the enzymes of choice for potential applications in the food, detergent, pharmaceutical, leather, textile, cosmetic, and paper industries. The most significant industrial applications of lipases have been found mainly in the food, detergent, and pharmaceutical sectors [3].

Lipases, as multipurpose biological catalysts, offer a favourable outlook for meeting the needs of several industries, such as food, beverages, textiles, leather, detergents, pharmaceuticals, and medicals [4]. The Lipase Market size is estimated at USD 448.78 million in 2024 and is expected to reach USD 645.18 million by 2029, growing at a CAGR of 7.53% during the forecast period (2024–2029) [Growth Trends & Forecasts 2024 - 2029]. The contributions of bacterial lipases, fungi, animals, plants, and algae are 45%, 21%, 18%, 11%, and 3%, respectively [5]. Many bacterial lipases are better studied than those in plants and animals are. Bacterial lipases exhibit distinct properties; they are active over a wide range of pH values and temperatures. The optimum pH of bacterial lipase ranges from 4.0 (*Rhodotorula pilimanae* lipase I) [6] to 10.0 (*Bacillus alcalophilus*) [7], and the temperature range is between 28°C (*S. haemolyticus*) [8] and 80°C (*B. thermoleovorans* ID-1) [9], whereas the molecular weight varies from 11 kDa (*B. thermoleovorans* CCR11) [10] to 176 kDa (*Rhodotorula pilimanae* lipase I) [6]. Whereas the fungal lipase pH ranges from 5.2 (*A. niger*) [11] to 11.0 (*Penicillium nitroaeducens*) [12], the temperature ranges from 25°C (*Fusarium solani*) [13] to 60°C (*Thermoactinomyces* sp.) [14], and the molecular weight of lipase varies from 27 kDa (*Aspergillus carneus*) [15] to 120 kDa (*Candida cylindracea*) [12]. Similarly, in plants and animals, the activity of lipase varies widely; the optimum pH is 5.0 (*Cucumis melo*) [16], 9.0 (*Castor bean*) [17], 4.5 (*Homo sapiens* gastric) [18], and 9.0 (*Scorpio maurus*) [19]. The optimum temperature of plants ranges from 25 (Castor bean) [17] to 60 (*Caesalpinia bonducella* seeds) [20], and for animals, it is 15 (*Oncorhynchus mykiss* trout) [21] and 40 (*Sus scrofa*) [22]. The molecular weight ranges from 40 kDa (rice bran) [23] to 143 kDa (*Triticum aestivum*) [24] in plants, and in animals, it is between 10 kDa (*Homo sapiens* gastric) [18] and 180 kDa (*Rattus norvegicus*) [25]. In algae, the lipase enzyme is reported in *Spirulina platensis* [26], *Botryococcus sudeticus* [27], *Chlorella vulgaris* [28], *Oscillatoria tenuis, Anabaena* sp., *Nostoc* sp., *Calorella ellipsoidea,* and *Chlorella vulgaris* [29]. However, they are not fully characterized, except *for Spirulina platensis* [26] and *Botryococcus sudeticus* [27].

In this study, *S. obliquus* was used as a lipase source for the first time, and the lipase was purified and characterized with respect to molecular weight, optimal temperature and pH, kinetic constants, and substrate specificity. The effects of

organic solvents, metal ions, detergents, and enzyme inhibitors on lipase activity were also investigated.

2. Materials and Methods

Media and strains

Experiments were carried out using *S. obliquus* or *Tetradesmus obliquus* (GenBank: KU170646.1), which were isolated in the laboratory. The microalgae were purified by the streak plate technique to obtain an axenic culture. The microalgae cultivated in TAP medium were used for both solid (0.5% agar) and liquid growth with shaking at 100 rpm. The algae were grown at room temperature under continuous light at 35–40 µmol/m^2/s [30].

Protein assay

Total protein and enzyme activity were determined using spectrophotometry (UV–Vis) and the p-nitrophenyl palmitate (p-NPP) hydrolysis method [31], [32], [33].

Lipase activity assay

Lipase activity was measured with a modified spectrophotometric assay with p-NPP as the substrate. Solution A contained pNPP (para-nitrophenylpalmitate) (90 mg) dissolved in ethanol (30 ml), and solution B contained Triton X-100 (2 g) and gum arabic (0.5 g) dissolved in 450 ml of buffer (50 mM Tris-HCl, pH 8.0). The assay solution was prepared by adding 1 ml of solution A and 9 ml of solution B dropwise to obtain an emulsion that remained stable for 2 h. The assay mixture contained 900 µl of the emulsion and 100 µl of enzyme solution. The liberated p-nitrophenol concentration was measured at 410 nm [27,34].

Lipase activity by the titrimetric method: Lipase activity was determined in a substrate solution with a ratio of arabic gum: 0.2 M phosphate buffer (pH 7): coconut oil of 2:1:1. All the components of the substrate solution were mixed until homogeneous. Afterward, 0.1 mL was removed and placed in a flask, after which 1 mL of 1 M $CaCl_2$ was added. The solution was mixed with 0.01 mL of crude enzyme, homogenized by vortexing, and incubated on a rotary shaker (150 rpm) at 30°C for 1 h. The reaction was stopped by the addition of a 5 mL of an acetone:ethanol (1:1) mixture after the incubation period, after which the mixture was homogenized by shaking. Titration was performed with 0.01 N NaOH until the solution turned pink. One unit (U) of lipase activity was defined as the amount of enzyme that was used to produce one µmol of fatty acids equivalent per minute per mL under specified assay conditions.

Lipase activity = $\Delta V \; x \; N \; x \; 1000/V \; sample \; x \; T$

where ΔV = V2 – V1. where V1 is the volume of NaOH as a control, V2 is the volume of NaOH as an experimental, N is the normality of NaOH, V is the volume of enzyme, and T is the incubation time [35].

Purification of lipase

Extraction and partial precipitation of lipase

The cells were harvested by centrifugation at 12,000 rpm for 30 minutes. The harvested cells were subsequently washed three times with w10 mM phosphate buffer (pH 7.0) fer (pH 7.0) at room temperature, after which 20 g of wet sample was suspended in 50 ml of 0.01 M phosphate buffer supplemented with 0.1 mM EDTA. Cell-free extracts were prepared by sonicating the suspension at 149 µm, with 20 seconds on and 10 seconds off cycles. The sonicated solution was centrifuged at 12,000 rpm for 30 min at 4°C, and the supernatant was used to determine the lipase activity. Ammonium sulfate precipitation of the cell-free extract was carried out at 4°C over a saturation range of 20–80% (w/v). The mixture of ammonium sulfate (different percentages) and the enzyme extract was incubated at 4°C for 1 h, followed by centrifugation at 12,000 rpm for 30 min.

Organic solvents (acetone) were added to the extract solution containing lipase such that a defined concentration (20–80%) was achieved. The precipitate was stabilized at 4°C for 1 h and then centrifuged at 12000 rpm for 10 min [36].

The precipitate was dissolved in 50 mM phosphate buffer at pH 7.0. Dialysis of this sample was carried out using Sigma cellulose tubing (molecular weight cut-off: 12–14 kDa) for 24 h with four buffer changes at 4°C [26].

Purification of lipase by ion exchange chromatography

The dialyzed sample was loaded onto a DEAE-cellulose column (1.2 cm × 15 cm) previously equilibrated with 50 mM phosphate buffer (pH 7). After the bed volume of the equilibration buffer was doubled, elution was performed with a linear gradient of 0 to 1.0 M NaCl in the same buffer at a flow rate of 60 ml/min. Fractions showing lipase activity were pooled and analysed [26].

Purification of lipase by gel filtration chromatography

The lipase-positive pooled samples were sorted, and the high lipase-positive fraction was applied to a Sepharose-6B fast flow column (1.2 cm × 30 cm), which was preequilibrated with 50 mM phosphate buffer (pH 7.00) and eluted with the same buffer at a flow rate of 30 ml/min. The fractions were collected and analysed for lipase activity [26].

Molecular weight determination by gel electrophoresis

Molecular weight and purity were analysed by SDS–PAGE and native polyacrylamide gel electrophoresis (native PAGE) using the method of Laemmli [37] on a 12.5% polyacrylamide gel at 150 V for 1 h. The reference proteins for molecular mass determination (Genei, Bangalore) were lysozyme (14.3 kDa), soybean trypsin inhibitor (20.1 kDa), carbonic anhydrase (29 kDa), ovalbumin (43 kDa), bovine serum albumin (66 kDa), and phosphorylase b (97.4 kDa). The protein bands were molecular weight markers (range: 97.4–14.3 kDa; Genei, Bangalore) and were detected by Coomassie Brilliant Blue R-250.

Determination of optimal pH, temperature and kinetic constants

The optimal pH of the lipase was determined by measuring activity over a pH range of 4–12 using Britton–Robinson buffer. The lipase activity of the samples in each buffer was measured using the standard assay as described previously. The optimal temperature for the purified lipase was determined by measuring activity across 20–80°C at a pH of 8.0 in 50 mM Tris-HCl buffer. The activity assays were carried out at different p-NPP concentrations (10–100 mM) to

determine the maximum reaction rate (Vmax) and Michaelis–Menten constant (km) of *S. obliquus* lipase [26].

Effects of metal ions, organic solvents, detergents and enzyme inhibitors on lipase activity

Various effectors, including 1 mM metal ions (Mg^{2+}, Ca^{2+}, Zn^{2+}, Fe^{3+}, Cu^{2+} and Mn^{2+}), 1% organic solvents (acetone, ethanol, methanol, isopropanol, n-butanol, chloroform diethyl ether and hexane), 0.1% detergents (SDS, Tween 20, guaiacol and Triton X-100) and enzyme inhibitors (EDTA), were added to the enzyme solution and incubated at room temperature for 30 min. Residual activity was measured by a lipase assay using a titrimetric method [35].

Determination of substrate specificity

Lipase activity on different oils (olive oil, sunflower seed oil, coconut oil, soybean oil, groundnut oil, palm oil, Mustard oil, and castor oil) was studied using a titrimetric lipase assay to determine the substrate specificities of the purified lipase [35].

3. Results and discussion

Purification of S. obliquus lipase

A summary of a typical purification of *S. obliquus* lipase is shown in Table 1. A lipase activity assay was used. The crude supernatant had a hydrolytic activity against p-NPP of 10.77 U/ml after 7 days of culture. The extract was purified by solvent and salt precipitation to isolate the lipase. To increase enzymatic activity, the solvent and salts were removed by dialysis. However, owing to the presence of chlorophyll in the ammonium sulfate-precipitated extract, the acetone-precipitated extract was used for the sequential application of ion-exchange chromatography (DEAE cellulose) and column chromatography (Sephadex G-100). The results showed that the lipase was precipitated by 80% saturated acetone, leading to a 0.82-fold increase in the crude enzyme extract, with a specific activity of 0.289 unit/mg. The final purified lipase after Sephadex G-100 chromatography had total yields of 12.95% and 6.79% (Table 1). The purified lipase was homogeneous when tested by SDS–PAGE. The molecular weight of the *S. obliquus* lipase was 36 kDa. The specific activity of the purified lipase was 2.381 U/mg protein.

Table 1: Flowsheet of the procedure used to partially purify lipase from *S. obliquus*

Purification Stage	Total Volume (ml)	Enzyme Activity (U/ml)	Protein Concentration (mg/ml)	Total Enzyme Activity (U)	Total Protein Concentration (mg)	Specific Activity (U/mg)	Purification Fold	Yield (%)
Crude	50	10.77	30.56	538.46	1528	0.352	1.00	100.00
Ammonium sulfate	15	9.56	43.12	143.40	646.8	0.222	0.63	26.63
solvent precipitation	15	14.18	49.07	212.70	736.05	0.289	0.82	39.50
Ion exchange chromatography	6	16.78	9.89	100.68	59.34	1.697	4.82	18.70
Column chromatography	4	17.43	7.32	69.72	29.28	2.381	6.76	12.95

Effects of pH and temperature on enzyme activity

The purified lipase tolerated a pH of 4.0–11.0 (Fig. 1a). The optimum pH for lipase activity is 8.0. Lipase activity was 32% of its maximum value when the pH was 6.0. The activity decreased significantly when the pH increased from 9.0 to 11.0, and approximately 11% residual activity was obtained at a pH of 8.0. However, somewhat different results were observed in *S. platensis*; the purified lipase could tolerate pH 5.0–8.0, and the maximum lipase activity was obtained at pH 6.5. *B. sudeticus* also has a wide range of pH tolerances, with significant enzyme activity measured from a pH of 5 to a pH of 11 to its optimum value of 10 [27].

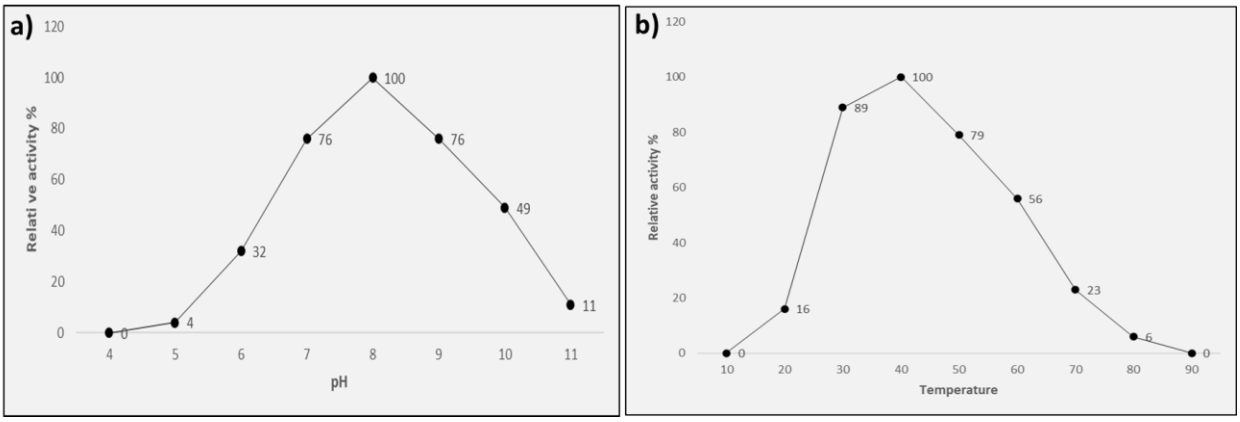


Figure 1 (a-b): Influence of (a) pH and (b) temperature on the hydrolysis of p-NPP by purified lipase

The purified lipase was active in the temperature range of 30–60°C, with maximal activity occurring at 40°C (Fig. 1 b). The activity decreased rapidly above 50°C, and no activity was detected at 90°C. These results were similar to those of S. platensis, whose activity ranged from 30 to 60°C, and the optimum temperature was 45°C [26]. In *B. sudeticus,* lipase is active over a broad range of temperatures from 4°C to 90°C and is highly active between 40°C and 70°C, with optimum activity occurring at 50°C [27]. Lipase from *Microbacterium* sp. also showed activity in the range of 10–90°C. The optimum temperature for *Microbacterium* sp. lipase was found to be 50°C [38]. *Yarrowia lipolytica* (40°C) [39] and *Aspergillus niger* (45°C) [40].

Determination of kinetic constants

The values of km and Vmax of the purified *S. obliquus* lipase, as calculated from the Lineweaver–Burk plot, were 2.15 mM and 46.72 µmol min−1 mg−1, respectively (Fig. 2). The results revealed that the km values of the lipase (p-NPP as a substrate) from *S. obliquus* were greater than those of *B. sudeticus* (2.02 mM) [27] and *S. platensis* (0.02 mM) [26], *Bacillus stearothermophilus* MC 7 (0.33 mM) [41], and *P. aeruginosa* (0.037 mM) [42] but lower than the km values of lipases from other sources, such as *A. niger* (7.37 mM) [40], *B. subtilis* FH5 (5.05 mM) [43], *Microbacterium* sp. (3.2 mM) [37] and *Bacillus flexus* (2.25 mM) [44]. The Vmax value is close to *that of B. sudeticus, which is* 33.00 µmol min-1 mg-1 [27].

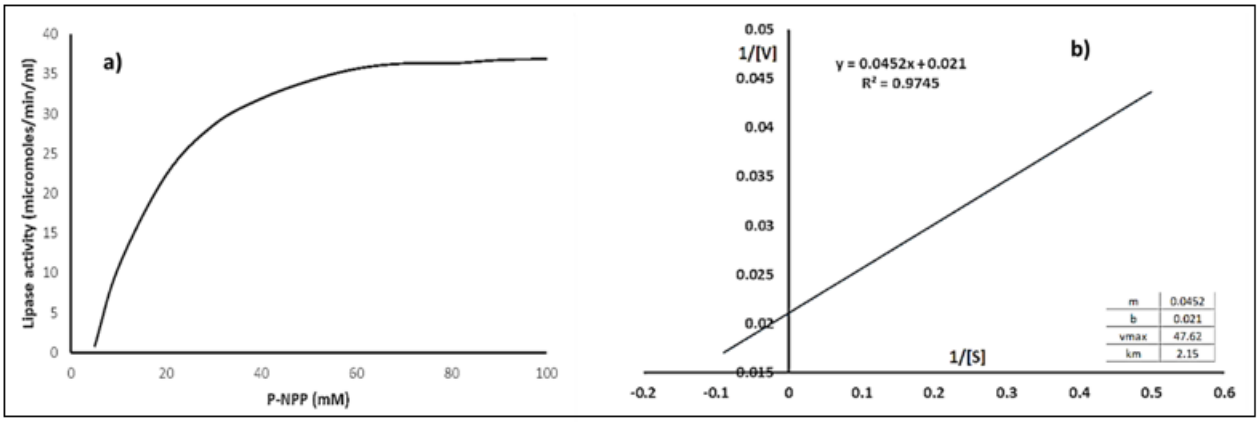


Figure 2(a–b): Kinetic studies of m *S. obliquus* lipase a) Effect of different concentrations of substrate on purified lipase and b) determination of the km value of lipase by Lineweaver–Burk plot

Determination of substrate specificity

S. obliquus lipase is active against a wide range of natural lipids, such as olive oil, sunflower seed oil, coconut oil, soybean oil, groundnut oil, palm oil, mustard oil, and castor oil (Fig. 3a). Olive oil is used as a standard source for the determination of lipase activity and is considered 100%. The lipase activity against different sources ranged between 84 and 91%, and the activity against castor oil was 54%. Similar results have been reported for *S. platensis* lipase, whose hydrolysis efficiency is high for olive oil, sunflower seed oil, soybean oil, palm oil, and mustard oil, but it has low hydrolysis efficiency for canola oil, peanut oil, cottonseed oil, and castor oil [26].

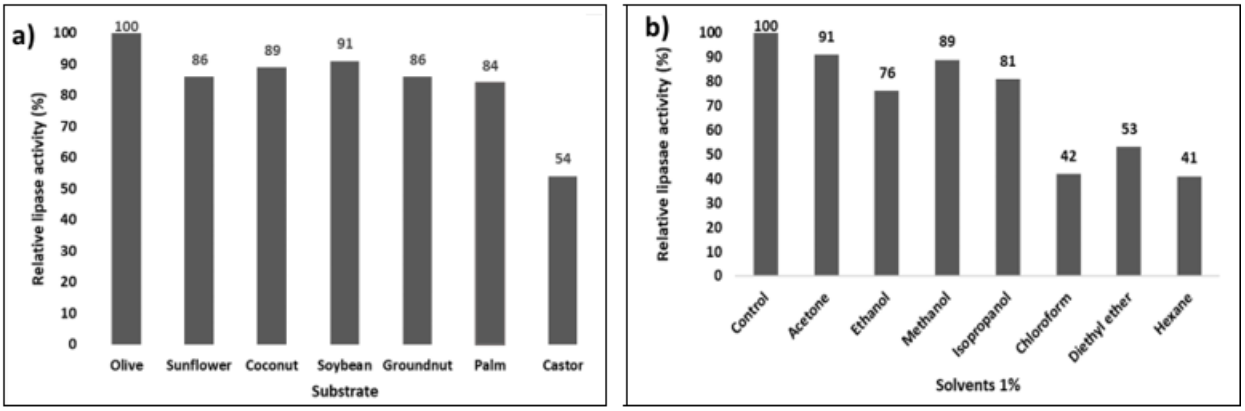


Figure 3(a-b): Influence of (a) substrate and (b) solvents on purified lipase

Effects of organic solvents, metal ions, detergents and enzyme inhibitors on lipase activity

The effects of organic solvents on lipase activity are depicted in Figure 3.b. Lipase was more stable in acetone (91%) than in the other tested organic solvents, ethanol (86%), methanol (89%), and isopropanol (81%), and was slightly inactivated in chloroform (42%), diethyl ether (53%), and hexane (41%). The effects of various kinds of metal ions on enzyme activity are shown in Figure 4a. Cationic ions, such as Ca^{2+} (93%), Zn^{2+} (94), Fe^{3+}(99), Cu^{2+}(89%), Mn^{2+} (83%), and Hg^{2+} (24%), tended to inhibit lipase activity, except for Mg^{2+} (112%). Similar observations have been reported for *Pseudomonas fragi* 22.39 lipase, which is inhibited by other cations and slightly activated by Ca^{2+} [45]. *S. platensis* lipase is activated by Mg^{2+}, Ca^{2+} and Zn^{2+} but inhibited by Li^{+}, Fe^{3+}, Fe^{4+}, Cu^{2+} and Mn^{2+} [26]. The effects of various types of detergents and inhibitors on enzyme activity are shown in Figure 4.b. The lipase was active in the presence of SDS (120%), Tween 80 (108%), Triton X-100 (104%), and EDTA (106%) and was inhibited by urea (18%) and guaiacol (91%). Similarly, *S. platensis* lipase is highly active in the presence of 0.1% Triton X-100 or 0.5 mM SDS and inhibited by 0.1% EDTA or 0.1% PMSF [26], whereas *B. sudeticus* lipase activity is activated by EDTA and PMSF and metal ions such as $FeSO_4$, $MnSO_4$, NaCl, $ZnCl_2$, and $ZnSO_4$ and inhibited by $CaCl_2$ [27].

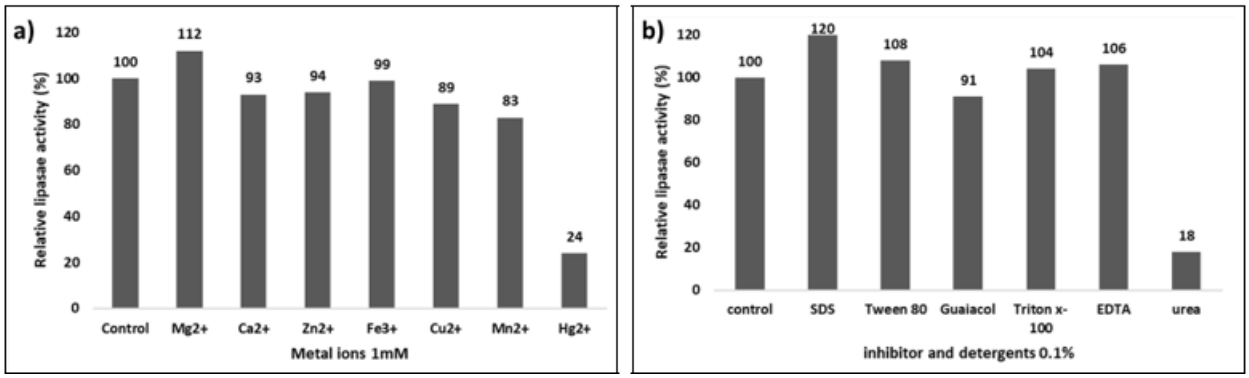


Figure 4(a-b): Influence of (a) metal ions and (b) detergents and inhibitors

4. Limitations and Future Aspects of the Study

Although the present study provides valuable insights into the purification and biochemical characteristics of intracellular lipase from *Scenedesmus obliquus* S6, several limitations should be acknowledged. First, the enzyme was purified using acetone precipitation followed by Sephadex G-100 chromatography, and the relatively low final recovery (12.95%) indicates that further optimization of the purification strategy may be necessary to improve enzyme yield and recovery. Second, the molecular mass of the purified lipase was determined to be approximately 36 kDa; however, detailed structural and molecular characterization was beyond the scope of the present study. Future investigations should therefore employ complementary approaches such as mass spectrometry, peptide sequencing, proteomic analysis, and molecular characterization of the corresponding lipase-encoding gene to establish its molecular identity and sequence characteristics.

The present study primarily evaluated enzyme activity under selected temperature, pH, metal-ion, detergent, chelator, and solvent conditions. However, important catalytic properties, including substrate specificity, kinetic parameters (Km and Vmax), catalytic efficiency, activation energy, and long-term operational stability, require further investigation. In addition, the mechanisms underlying the activation or inhibition of the enzyme by different metal ions, detergents, and chemical agents remain to be elucidated. Detailed structural studies could provide insights into the relationship between the molecular architecture of the enzyme and its observed biochemical stability.

Although the biochemical properties of *S. obliquus* lipase indicate potential for industrial applications, particularly in lipid bioconversion and biodiesel-related processes, the present study did not directly evaluate its performance in biodiesel production. Therefore, further research should investigate its catalytic efficiency toward relevant lipid substrates and compare its transesterification performance with commercially available and other microbial lipases. Enzyme immobilization should also be explored to improve reusability, operational stability, resistance to organic solvents, and suitability for continuous bioprocessing. Optimization of reaction conditions, including substrate concentration, enzyme loading, water content, alcohol-to-oil ratio, reaction temperature, and reaction time, would be important for evaluating its practical biodiesel-producing potential.

Future studies should also investigate large-scale cultivation of *S. obliquus* S6 and optimization of culture conditions to enhance lipase production. The use of inexpensive substrates, nutrient optimization, and integrated algal biomass utilization could improve the economic feasibility and sustainability of enzyme production. Furthermore, techno-economic assessment and life-cycle analysis would be valuable for determining whether algal lipase production and its subsequent application in biodiesel synthesis can provide meaningful environmental and economic advantages over conventional enzyme sources.

The present findings establish *S. obliquus* S6 as a promising source of an alkaline lipase with broad pH tolerance and useful biochemical properties. Future studies integrating molecular characterization, enzyme engineering, immobilization, process optimization, scale-up, and direct application testing will be essential to translate these laboratory findings into commercially viable biotechnological applications.

5. Conclusion

Lipases are widely distributed among microorganisms, plants, and animals and have attracted considerable attention because of their broad industrial and biotechnological applications. However, algal lipases remain comparatively underexplored, despite the demonstrated lipolytic activity of several algal species. To the best of our knowledge, only limited studies have reported the isolation, purification, and biochemical characterization of algal lipases, making the present investigation of particular significance.

In this study, an intracellular lipase from the freshwater microalga *Scenedesmus obliquus* S6 was successfully purified using 80% (v/v) acetone precipitation followed by Sephadex G-100 chromatography. The purified enzyme exhibited a 6.79-fold purification, a total recovery of 12.95%, and a specific activity of 2.381 U/mg protein, with an apparent molecular mass of approximately 36 kDa. The enzyme demonstrated a broad operational range, retaining activity between 30 and 60°C and across pH 4.0–11.0, with optimum activity at 40°C and pH 8.0. These characteristics indicate that the enzyme possesses substantial thermal tolerance and broad pH adaptability, including activity under mildly alkaline conditions.

The purified lipase also displayed differential responses to metal ions, detergents, chelating agents, and organic solvents. Mg^{2+} enhanced enzyme activity, while SDS, Tween 80, Triton X-100, and EDTA maintained or increased relative activity. Conversely, several organic solvents and metal ions caused varying degrees of inhibition, demonstrating the sensitivity of the enzyme to its chemical environment. Collectively, these biochemical characteristics highlight the functional versatility of *S. obliquus* lipase and distinguish it as a promising candidate for further investigation.

Overall, the findings establish *S. obliquus* S6 as a valuable source of an alkaline lipase with broad pH tolerance and activity under diverse physicochemical conditions. The enzyme's biochemical properties suggest potential utility in industrial biotechnology, including applications in lipid bioconversion and biodiesel-related processes. However, its practical applicability should be validated through further studies involving enzyme immobilization, substrate specificity, catalytic efficiency, operational stability, and direct evaluation in biodiesel production systems. Such investigations could help establish *S. obliquus* as a sustainable source of commercially relevant lipases and expand the biotechnological potential of freshwater microalgae.

Declaration of competing interests

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

Disclosure statement

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Data availability statement

All data documented in the manuscript.

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