Renewable Energy 153 (2020) 919–934

Contents lists available at ScienceDirect

Renewable Energy

journal homepage: www.elsevier.com/locate/renene

# Novel biocatalyst for optimal biodiesel production from diatoms

G. Saranya [a, b], T.V. Ramachandra [a, b, c, *]

[a] Energy & Wetlands Research Group, Center for Ecological Sciences [CES], Indian Institute of Science, Bangalore, Karnataka, 560 012, India
[b] Centre for Sustainable Technologies (astra), Indian Institute of Science, Bangalore, Karnataka, 560 012, India
[c] Centre for Infrastructure, Sustainable Transportation and Urban Planning [CiSTUP], Indian Institute of Science, Bangalore, Karnataka, 560 012, India

ARTICLE INFO

*Article history:*
Received 12 September 2019
Received in revised form
15 January 2020
Accepted 13 February 2020
Available online 18 February 2020

*Keywords:*
Diatom oil
Fungal lipase
Biocatalyst
Enzymatic transesterification
Microalgal biodiesel

ABSTRACT

Fatty acid methyl ester (biodiesel) has been derived from oil present in algae through transesterification using catalysts of acids, base, supercritical fluids, etc. These catalysts are corrosive and have been posing challenges of contaminating the environment necessitating environmentally friendly and biodegradable catalysts such as enzyme (lipase) based biocatalysts. In this study, fungal strains (endophytic/free spores) were isolated from an estuarine ecosystem and screened for extracellular lipase activities. A novel fungal strain *Cladosporium tenuissimum*, identified through molecular technique exhibited higher lipolytic activity among the isolates. The crude lipase extracted from fungus was subjected to ammonium sulphate precipitation and purification using Superdex 200 gel filtration chromatographic system. The molecular weight of purified lipase was found to be ~46 KDa and a specific activity of 37.2 U/mg. Lipase activities attained stability and reached maximum at 60 °C temperature and pH of 6. The purified enzyme was used as a biological catalyst for enzymatic transesterification of oil obtained from an indigenously isolated salt tolerant diatom *Nitzschia punctata*. Spectroscopic analysis on fatty acids and Fatty Acid Methyl Esters derived from diatom exhibited similarities in specific functional groups between algal oil and biodiesel. Comparisons on biodiesel yield estimation and FAME compositions of enzyme catalyzed, acid catalyzed biodiesel assessed through gas chromatographic techniques revealed a higher efficiency (87.2 ± 0.47%) of biocatalysts compared to conventional acid catalyst (83.02 ± 0.35%) exhibiting potential scope for large scale application of environment friendly biocatalysts to enhance the conversion performances of the transesterification process.



## 1. Introduction

Energy is a pivotal resource for the economic development of a region [1], a resource that is confronted with far exceeding demands than supply at recent times due to speculated rapid industrialization and sophisticated living [2]. Adverse and irreversible effects due to global warming and consequent changes in climate make microalgae derived biofuels as the most attractive and sustainable energy options [3]. Algal biodiesel is renewable [4] with its feedstock having the ability to sequester atmospheric $CO_2$, which enhances the scope of sustainable energy option [5]. Algae as a biodiesel feedstock include an array of advantages like higher photosynthetic efficiency (12.6%), efficient $CO_2$ sequestration capability, potential to bioremediate contaminated waters and non-arable lands [6,7], with the proven higher oil content [8] and algal biomass productivity. The maximum theoretical algal biomass productivity reported so far in a region of higher solar insolation is around 100–120 g/m$^2$/day [9]. Algae stores fats and lipids in the form of triglycerides which is high quality and high-volume raw material. Most oleaginous microalgae accumulate 20% total lipids on dry cell weight and this increases up to 50% when algae are subjected to stress conditions [10]. Oil is being extracted from algae through transesterification process using chemical catalysis. There is a trade-off between the alkoxy group of an ester with a methyl group in most transesterification reactions [11]. Methanol or ethanol is used as a co-reactant (acyl acceptors) for algal derived oil but methanol is mostly preferred due to its lesser cost when compared to ethanol. Major catalysts that are in extensive use so far in the transesterification process are either of acid or alkali. The most commonly used acid catalysts are sulfuric acid and hydrochloric acid in its diluted forms while alkali catalyzed reaction uses

* Corresponding author. Energy & Wetland Research Group, CES TE 15, Centre for Ecological Sciences, New Bioscience Building, Third Floor, E-Wing, [Near D-Gate], Indian Institute of Science, Bangalore, 560012, India.
*E-mail addresses:* tvr@iisc.ac.in, energy.ces@iisc.ac.in (T.V. Ramachandra).
*URL:* http://ces.iisc.ernet.in/energy

https://doi.org/10.1016/j.renene.2020.02.053
0960-1481/

sodium hydroxide or potassium hydroxide. The major drawbacks of acid- or alkali-based) catalyst assisted transesterification reactions are (i) low yield and purity due to unwanted side reactions, (ii) high energy requirements, (iii) higher costs involved in by-product (glycerol) separation, and (iv) need for neutralization and wastewater treatment, post-reaction completion [12]. The use of enzymes as biocatalysts for transesterification [13] is an emerging technique compared to conventional acid/alkali catalysis for biodiesel production. Enzymes synthesized by fermentation of biobased materials [14] are naturally occurring biocatalysts,. Lipolytic enzymes play a crucial role in turn-over and mobilization of lipids, a major component of earth's biomass from one organism to another [15]. Microorganisms such as bacteria and fungi produce biosurfactants are known to solubilize lipids [16]. Moreover, when enzymes are used as biocatalysts during transesterification of algal oil, it renders a cleaner and an environmentally friendly option with an added advantage for third-generation (microalgal) biofuels. Among other enzymes, lipase (triacylglycerol acyl hydrolases, EC 3.1.1.3) and esterase (E.C. 3.1.1.1) are the two major classes of lipid hydrolytic enzymes belonging to α/β hydrolase family that are being considered as promising industrial biocatalysts [17]. Esterase enzyme catalyzes the hydrolysis of shorter chain length fatty acid esters (<C8), while lipase catalyzes triacylglycerols which are of longer chain lengths (>C8) [18,19]. Lipase enzymes are categorized into three different classes based on the type of substrates: (i) lipase with regio- or positional specific lipolytic active sites, (ii) fatty acids specific lipases, (iii) highly specific to only certain acylglycerols present in oils [20]. These lipolytic enzymes are receiving considerable demand as potential industrial biocatalysts due to its manifold applications in dairy, food, detergents, fats and oil, organic synthesis, biodiesel, agro-chemicals, new polymeric materials [21], paper and pulp, leather, fine chemicals, cosmetics, pharmaceuticals [22–25] and various environmental applications including soil bioremediation and biodegradation of environmentally toxic pollutants such as phenolic compounds and endocrine disruptors [26]. Although lipases are ubiquitous and produced by most microorganisms, plants and animals, the most industrially exploited lipase sources are of microbial origin isolated commonly from bacteria and fungi [27,28]. Compared to other extraction sources, microbial lipases possess several advantages such as shorter cycling time, less expensive and are easily adaptable to grow and immobilize on any inexpensive solid media (substrates). They often fetch higher yields and also are compatible to genetic manipulations. Enzyme catalysts require milder ambient conditions, compared to chemical catalyzed reactions for its effective operation leading to a major cut-down in energy expenditure and hence the operational costs. Other advantages of enzymatic reactions include high selectivity towards substrate with the capability to esterify triglycerides and free fatty acids in a single step, thus producing high-quality byproduct (glycerol) with no additional costs involved in byproduct separation and recovery. Enzymes are highly specific to substrates, thus eliminating unwanted side reactions and the need for post-reaction byproduct separation. Moreover, enzymatic reactions are environment-friendly without posing any hazards during disposal [29]. There have seen enormous efforts during the past decade focusing on lipase enzyme production and characterization for diverse applications. Biodiesel production using lipase as a biocatalyst is an emerging area of research with its application already standardized for first and second-generation biodiesel feedstocks such as sunflower oil [30], Jatropha oil [31], soybean oil [32], palm oil [33]. Recent researches focus on using lipase as biocatalyst [20,34–37] in the enzymatic conversion of microalgal oil into biodiesel.

### 1.1. Motivation for the study

Algae are primary producers in aquatic ecosystems and are emerging as promising biodiesel feedstocks due to the presence of proven higher oil content in the form of triglycerides. Algae based biodiesel have an array of advantages like viable replacement to fossil fuels, assured stock availability, efficient $CO_2$ sequestration capability, remediation and treatment of water etc. Fatty acid methyl ester (biodiesel) is derived from oil present in algae through transesterification. Catalysts of acids, base, supercritical fluids, etc., are being used to maximize the conversion of oil into biodiesel. These catalysts are corrosive and have been posing challenges of contaminating the environment necessitating environmentally friendly and biodegradable catalysts such as enzyme (lipase) based biocatalysts. Industrially important enzymes extracted from indigenous sources are least explored, especially for biofuel production. Exploitation of cellulase and lipase for biofuel production would greatly reduce the environmental burden imposed by conventional chemical catalysts. In the current study, extracellular lipase extracted from an indigenously isolated fungal strain *Cladosporium tenuissimum* was used as a biocatalyst to derive biodiesel from a salt-tolerant diatom *Nitzschia punctata* (microalga). Crude extracellular lipase was purified using gel filtration-based size-exclusion chromatographic system. The purified enzyme after characterization was used as a biological catalyst for transesterification of microalga derived oil. In addition, biodiesel (FAME) was derived using a conventional acid catalyst. Biodiesel derived from the acid catalyst and enzyme catalyst-based transesterification were assessed for FAME yields to understand the relative performances and efficiencies of the catalysts possessing different chemical properties.

## 2. Materials and methods

Conventional biodiesel production protocol is given in Fig. 1 and Fig. 2 outlines the method followed in the present endeavor to optimize biodiesel production through novel biocatalyst as well as a conventional chemical catalyst.

### 2.1. Isolation, screening and identification of lipase producing fungi

Indigenous fungi that are efficient in extracellular lipase production, were explored in natural environments. Endophytic fungi (from root, shoot, bark and leaf of a mangrove plant) and free spores (from sediment and water column) were isolated from samples sourced from a backwater region (14°24′58.14"N, 74°24′30.30"E,

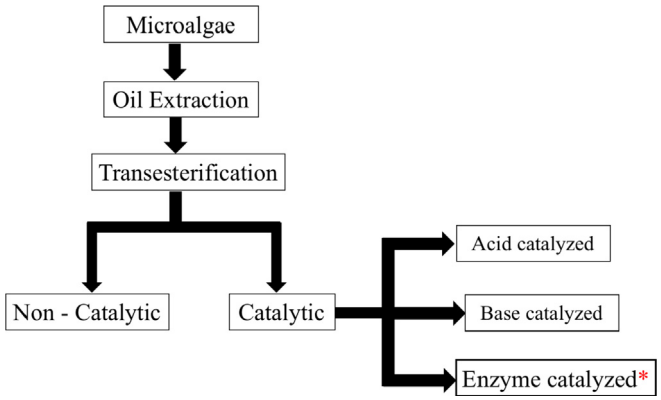


**Fig. 1.** Process outline of conventional algal biodiesel production.
* - protocol explained in detail in Fig. 2.

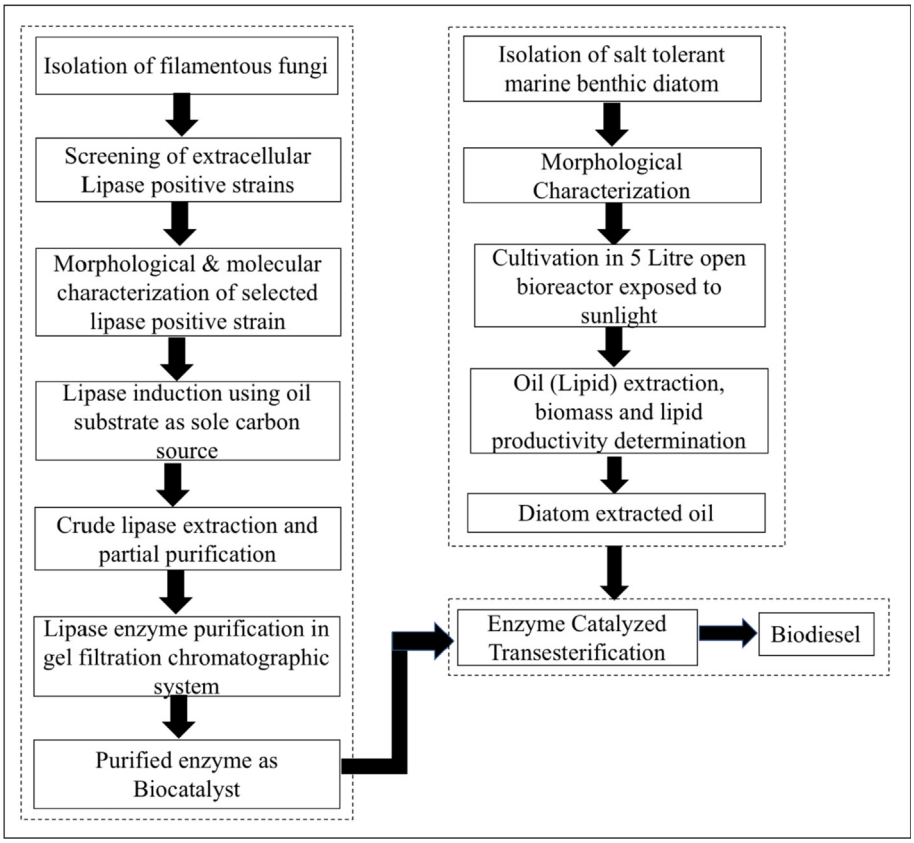


**Fig. 2.** Flowchart describing the workflow to optimize biodiesel production through novel biocatalyst.

with a salinity of 35 ppt) in Uttara Kannada district along the west coast of India. The collected samples were plated in the Potato Dextrose (PD) agar under aseptic conditions. Twenty-four fungal strains (CS1 – CS24) belonging to different genera were isolated during initial plating which was further sub cultured to obtain pure fungal isolates. These fungal isolates were subjected to multiple screening tests on agar plates for lipase using tween 80 and olive oil as sole carbon sources substituted with $CaCl_2.2H_2O$ and phenol red as extracellular lipase indicators respectively. Phenol red/olive oil agar plate was prepared using chemicals with composition (g/L) of: 0.01% (w/v) phenol red, 2% (w/v) agar, 1% (v/v) olive oil, 1% (w/v) $CaCl_2$ (anhydrous) with pH adjusted to 7.2 using 10 M NaOH [38]. Tween 80 plates were prepared as follows: 1% peptone, 0.5% NaCl, 0.01% $CaCl_2.2H_2O$, 2% agar and 1 ml (v/v) tween 80 [39]. The screening tests resulted in eight lipase positive strains (CS1 – CS8), and further one single strain (CS4) was screened based on the observation of the highest zone of clearance.

### 2.2. Morphological and molecular characterization of fungi

The morphological structure of the selected strain was studied after staining the fungus CS4 with lactophenol cotton blue and observing in the high-resolution phase-contrast optical microscope (Olympus BX51) under 40 × magnification. The fungal strain was also viewed under a scanning electron microscope (JEOL JSM IT-300) for better visualization of structural morphology. Further, the selected strain was subjected to 18S rRNA based molecular identification after extracting genomic DNA using DNeasy plant maxi extraction kit (Qiagen Inc., USA) and the extracted DNA were amplified using PCR with ITS1F (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS 4R (5′-TCCTCCGCTTATTGATATGC-3′) desalted 20 nm oligonucleotide universal primers. A single discrete PCR amplicon band of 500 bp was observed when resolved on an agarose gel. The PCR amplicon was then purified to remove contaminants and subjected to forward and reverse DNA sequencing reactions along with forward and reverse primers using BDT v3.1 cycle sequencing kit on ABI 3730xl Genetic Analyzer. The consensus sequence of 18S rRNA genes generated using forward and reverse sequences were subjected to nucleotide BLAST in National Centre for Biotechnology Information (NCBI) GenBank database to identify the strain based on maximum identity score. The first ten sequences that showed higher statistical significance with the strain of interest were selected and aligned using CLUSTAL-W multiple alignment software. The phylogenetic tree was constructed by multiple sequence alignment with the neighbour-joining method using MEGA software version 7. The 18S rRNA based molecular identification revealed a maximum homology of the strain CS4 to *Cladosporium tenuissimum* identified hereafter as *Cladosporium* sp. CS4.

### 2.3. Inoculum and lipase enzyme production medium

The *Cladosporium* sp. CS4 was inoculated in PD broth liquid medium (potato (infused form) 20% (w/v) and dextrose 2% (w/v)) at 28 °C for 7 days. After 7 days, the cell pellets formed a mat over the liquid layer were collected by centrifugation at 11,392×*g* for 20 min at 4 °C. The cell pellets were collected, which was used as inoculum for lipase production medium consisting of ingredients (g/L): $KH_2PO_4$ - 2.0, bacto-peptone - 5.0, yeast extract - 1.0, $NaNO_3$ - 0.5, KCl - 0.5, $MgSO_4{\cdot}7H_2O$ - 0.5, olive oil - 10.0, pH adjusted to 5.5 [40]. Cultivated with a culture volume of 1 L for 5 days at 28 °C. Then, the crude extract was separated from the mycelial mat by centrifugation at similar operating conditions for inoculum

preparation. The cell-free supernatant obtained after centrifugation with subsequent concentration and purification was used as a lipase enzyme source.

### 2.4. Enzyme concentration and purification

The cell-free supernatant was saturated with ammonium sulphate at a final concentration of 80% saturation with constant and slow stirring at 4 °C, followed by centrifugation at 18,900×*g* for 20 min at 4 °C [41]. The pellet obtained after centrifugation was dissolved in 50 mM Tris HCl pH 7.0 and dialyzed against 50 mM Tris-Chloride buffer (pH 7.2) with 0.1 M NaCl [42] at 4 °C overnight in a dialysis tubing having Molecular Weight Cut-Off (MCWO) of 3.5 KDa (Sigma-Aldrich). The concentrated enzyme after dialysis was subjected to a subsequent purification step that involved size exclusion-based gel filtration chromatography in a pre-equilibrated Superdex 200, 10/300 GL (GE Healthcare) with an elution rate of 0.15 mL/min. The eluted enzyme fractions (1 mL each) were collected in an AKTA pure 25 (GE Healthcare) FPLC collector. The collected enzyme fractions were subjected to protein estimation spectrophotometrically using Braford protein assay. The fractions that exhibited the highest protein content was subjected to lipase assay test and lipase active fractions were pooled and dialyzed extensively in 50% glycerol-based dialysis buffer containing 50 mM Tris-Chloride and 0.1 M NaCl with pH 7.2 and stored at 4 °C until use. The dialyzed fractions were run on SDS- PAGE to evaluate lipase purity.

### 2.5. Lipase activity determination

The rate of release of p-nitrophenol (p-NP) was used as a proxy to measure lipase activity through a spectrophotometer, using p-nitrophenol palmitate (p-NPP) as a synthetic lipid substrate [43]. The substrate solution was prepared according to Ref. [44] with the required modifications. Briefly, the substrate solution was prepared using 30 mg of p-NPP (sigma, AR grade) dissolved in 10 mL 2-propanol and mixed with 90 mL emulsifying solution containing 0.1% acacia (Arabic gum) and 0.4% Triton X-100, which resulted in a final substrate concentration of 0.8 mM of p-NPP. A volume of 100 μL cell-free supernatant was added to 100 μL of 0.5 M citrate phosphate buffer, pH 6.0 and 900 μL of substrate solution. The reaction was carried out in Eppendorf tubes in an incubator shaker maintained at 37 °C for 30 min under a rotation speed of 120 rpm. In order to stop the reaction, 0.1 mol $L^{-1}$ $Na_2CO_3$ [45,46] was used as stop reagent in the ratio of 1:1 (stop reagent: reaction media volume). Then the final reaction mixture was centrifuged at 4500 rpm for 5 min and the clear supernatant obtained was read at a wavelength of 410 nm using a microplate reader in order to quantify the amount of p-NP released. One unit (U) of enzyme activity is defined as the amount of enzyme that liberated 1 μmol of p-NP per minute at standard assay conditions. Lipase activity was determined by the formula derived using Beer-Lambert's law as explained in Ref. [38] where enzyme activity (U $ml^{-1}$) = ΔA.V/ε.t.v. ΔA: change in absorbance of enzyme sample and the blank over time; V: total reaction mixture volume (ml); ε: molar extinction coefficient in $\mu M^{-1}$ $cm^{-1}$; t: incubation time in minutes; v: volume in ml of enzyme used in the reaction.

### 2.6. Determination of size, purity and protein concentration of lipase enzyme

The molecular weight of the extracted enzyme and its purity was determined using Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS PAGE) as briefed by Refs. [47,48] on a 10% polyacrylamide gels. Unstained standard protein molecular weight markers (10–200 KDa) (Thermo Scientific) was used as a reference to determine the molecular weight of purified protein [49]. Silver staining was used to visualize protein bands on SDS PAGE gel [50]. Protein concentration was measured using Bradford protein assay with Bovine Serum Albumin (BSA) of increasing concentrations as standard at a wavelength of 595 nm in VERSA max tunable microplate reader (CARE Biosystems, Mumbai, India). Bradford reagent (5 × ) was prepared as follows in (g $L^{-1}$) - 0.05% Coomassie Brilliant Blue (CBB) G-250, 23.5% (v/v) of methanol and 50% (v/v) of 85% phosphoric acid which was diluted to 1 × concentration before protein assay analysis.

### 2.7. Characterization of purified lipase

#### 2.7.1. Effect of pH and temperature on lipase activity and stability

The lipase enzyme activities at varying pH ranges were measured at standard assay conditions with varying pH buffers of 50 mM concentration ranging from pH 3.0 – pH 10.0 using p-NPP as synthetic lipid substrate. The buffers used for pH optimization were as follows: Citrate phosphate (pH 3.0–6.0), Tris-HCl (pH 7), Potassium-phosphate (pH 8.0), Glycine-NaOH (pH 9.0–10.0). pH stability was determined by preincubating the enzyme aliquots with respective buffers for a period of 24 h at 4 °C [40]. Residual enzyme activity of the preincubated enzyme aliquots was measured by adjusting the pH to 7.0 and post-incubation of reaction mixture at 60 °C for 30 min after introducing p-NPP substrate. Temperature optimization of the enzyme was carried out by evaluating the enzyme activity at various incubation temperatures ranging from 30 °C to 80 °C (with increments of 10 °C) at pH 6.0. For determining thermal stability, the enzyme aliquots were pre-incubated with pNPP substrate at pH 6.0 under different temperatures for a period of 30 min and the residual enzyme activity was determined after post-incubation of respective enzymes at 60 °C for 30 min and assayed following standard protocols. Relative and residual activity expressed as a percentage (%) is determined by the ratio of activity at each temperature and pH range to that of the maximum recorded lipase activity.

$$\textit{Relative activity}\ (\%) = \frac{\textit{Activity}}{\textit{Maximum Activity}} \times 100 \qquad (1)$$

#### 2.7.2. Effect of surfactants on lipase activity

The effect of various surfactants on purified lipase was determined after incubating the enzyme aliquots with 0.125%, 0.25%, 0.5% and 0.75% (w/v) of SDS and similar concentrations in (v/v) of Triton-X, tween 20 and tween 80 at 37 °C without substrate for 1 h. After 1 h, the residual activity was determined after adding pNPP substrate and incubating the reaction mixture at 60 °C for 30 min with respect to control [40].

### 2.8. Isolation, cultivation & harvesting of microalgae

Indigenous diatom strains that are tolerant to higher salinities and capable of adapting to extreme environmental conditions were isolated and sediments samples were collected from a salt pan situated in vicinity to downstream of the Aghanashini estuary, Uttara Kannada, Karnataka, India (14°32′48.84"N latitude and 74°20′46.12"E longitude). The collected samples were subjected to serial dilution, agar plating and subsequent sub culturing of pure diatom isolate. The diatom isolates were later inoculated in F/2 medium [51] and sequentially scaled up in a 100 mL Erlenmeyer flask with 50 mL working volume under constant exposure of cells to a light intensity of 210 μmol $m^{-2}$ $s^{-1}$ with 12:12 h light-dark

cycles. An aliquot of grown algal biomass was then subjected to acid digestion following standard protocols [52] and the acid-treated sample was subjected to morphological observation and imaging using a high-resolution phase contrast microscope Olympus BX51 as well as Scanning Electron Microscope (SEM) in JEOL JSM-IT-300 with dry silicon drift detector (EDAX) and accelerating voltage up to 30 KV under a scanning magnification of 1000 × to facilitate observation of striae and accurate diatom valve measurements. The isolated diatom was identified as *Nitzschia punctata* (*Nitzschia* sp.) based on morphological characterization through high-resolution imaging and comparison with standard diatom taxonomical identification keys [53,54] The stock cultures of *Nitzschia* sp. were maintained in F/2 medium with routine sub culturing in filtered and sterilized estuarine water of salinity 35 ppt. Growth experiments were conducted on the rooftop with natural light (sunlight), 12: 12 h light/dark period after adding the inoculum of pure *Nitzschia* sp. having an approximate cell density of $1 \times 10^6$ cells/ml in 10 L translucent plastic tub with 5 L working volume. The cultures were maintained at ambient temperature for nine days with periodic mixing. At the end of the growth period, the diatom cells were harvested by siphoning the spent culture medium as the diatom cells sink and settle out in stationary cultures. The harvested biomass was washed twice with double distilled water, centrifuged at 3500×*g* [55] for 10 min and oven-dried at 85 °C overnight. The biomass productivity (mg $L^{-1}$ $d^{-1}$) of the dried algal biomass was calculated using equation (2).

$$\text{Biomass productivity (mg L}^{-1}\text{ d}^{-1}) = (C - C_0)/T - \quad (2)$$

where C is the final biomass concentration of algae after harvest, $C_0$ is the initial biomass concentration at the time of inoculation. T is the culture period in days.

### 2.9. Microalgal oil extraction and biodiesel production

#### 2.9.1. Oil extraction and quantification

The cultivated and harvested diatom (microalgal) biomass was oven-dried at 85 °C overnight and pulverized to a fine powder using mortar and pestle. Oil (lipid) extraction was performed on dried and pulverized algal biomass by following the modified Folch method [56]. The algal biomass was ultrasonicated at 35 kHz frequency at 45 °C for 15 min after adding chloroform and methanol in the ratio of 2:1 (v/v). The ultrasonicated sample was treated with 0.8% NaCl solution to enable a clear phase separation of aqueous and organic phases with cell debris at the interface between the two phases resulting in a solvent mixture ratio of (2:1:0.8) Chloroform/methanol/water. The lower organic phase consisting of lipids that are dissolved in chloroform was separated using separating funnel and the procedure was repeated twice to ensure maximum lipid recovery. The lipids thus extracted were evaporated under vacuum using a rotavap rotary vacuum evaporator (Model: PBV 7D) under reduced pressure and the oil obtained were weighed gravimetrically by dividing the lipid content obtained in mg with the biomass used for lipid extraction in terms of dry cell weight (dcw) to obtain lipid content (% dcw). Oil yield is calculated using equation (3).

$$\text{Oil yield } (\%) = \frac{\textit{mass of oil extracted } (mg)}{\textit{mass of algal biomass used } (mg)} \times 100 \quad (3)$$

#### 2.9.2. Fatty acid characterization

Fatty acids of lipids extracted in a 250 mL Soxhlet extraction unit, fitted with a reflux condenser was characterized using GC-MS. A known quantity of algal biomass (~500 mg) was taken in a cellulose extraction thimble and refluxed using hexane as a solvent in Soxhlet extractor for 5 h at 50 °C. The extracted fatty acids in hexane was concentrated using rotary vacuum evaporator under reduced pressure and characterized using GCMS: gas chromatography (Agilent Technologies 7890A GC, single quadruple analyser) – mass spectroscopy (Agilent 5975C inert MSD with triple-axis detector) model with helium as inert carrier gas and temperature at 35 °C for 2 min, with a ramp input rate of 35 °C–300 °C at 20 °C per min with final hold time of 5 min at 300 °C. The operating conditions were set with an initial solvent delay of 4 min [57]. The GC-MS generated peaks were interpreted through AMDIS data analysis software and the organic compounds eluted were matched with spectral mass spectroscopy database NIST V11.

#### 2.9.3. Acid-catalyzed transesterification

Aliquots of a known quantity of diatom extracted lipid (~20 mg) were subjected to acid-catalyzed transesterification. The reaction was carried out in a water bath connected to a reflux condenser and the reaction temperature was set at 80 °C until a reaction time of 2.5 h. A 2 wt% $H_2SO_4$ was used as a catalyst along with 2.5 ml of methanol as a co-reactant [58]. On reaction completion, the mixture was cooled to room temperature and phase separation was induced using hexane and distilled water in the ratio of 2:1. The upper phase containing Fatty Acid Methyl Ester (FAME) in hexane was collected in a sterile glass vial and the traces of water was removed by adding anhydrous sodium sulphate and stored in an Eppendorf tube for FAME composition analysis.

#### 2.9.4. Lipase catalyzed transesterification

A known quantity of lipid (~5 mg) extracted from 10 mg of pulverized algal biomass was taken in a sterile screw-capped vial and was added with 50 mM Tris-HCl buffer pH 6.0 in the ratio of 0.5:1 and purified lipase was added to about 10% by volume of the lipid substrate taken for analysis [59]. The methanol and substrate concentrations were maintained in a ratio of 6:1 [20]. The reaction was carried out at 40 °C for 48 h with constant shaking at 150 rpm. Methanol was added in parts at 0 h, 12 h and 24 h to avoid lipase inhibition to higher concentrations of methanol [60]. The FAME component analysis was carried out by collecting the upper organic phase containing biodiesel dissolved in hexane after visible phase separation on reaction completion.

#### 2.9.5. Quantification of biodiesel and conversion efficiency estimation

Biodiesel (FAME) composition and the variation in FAME yield between acid and enzyme-catalyzed transesterification reaction was quantified using peaks observed in GC-MS. The mass spectroscopy (ms) source and quadruple temperatures were 230 °C and 150 °C respectively. The program for oven operating conditions was set at 50 °C initially for a hold time of 2 min, then increased at a ramp heating rate of 10 °C/min until 280 °C with a holding time of 4 min. Methyl laurate ($C_{13}$) was used as an internal standard. FAME composition was determined in terms of percentage of each FAMEs out of the total present in the sample. The peaks were analysed using AMDIS data processing tool and FAME compounds were identified and matched using NIST V11 Mass spectral search program. The percentage conversion of fatty acids into corresponding fatty acid methyl esters (FAME) was estimated using equation (3) [59,61].

$$\text{Conversion } (\%) = \frac{m_{ester}}{3 \times \left(\frac{m_{oil}}{MW_{oil}}\right) \times MW_{ester}} \times 100 \quad (4)$$

where $m_{ester}$ indicates the weight of ester in g and $m_{oil}$ indicates weight of oil in g. $MW_{oil}$ and $MW_{ester}$ is calculated using equations (4) and (5).

$$MW_{oil} = 3 \times \sum_i (MW_i \times \%M_i) + 38 \quad (5)$$

$$MW_{ester} = \sum_i (MW_i \times \%M_i) + 14 \quad (6)$$

where $MW_i$ is the molecular weight of each of the fatty acid peaks obtained in GC-MS and $\%M_i$ corresponds to the percentage of fatty acids recorded in GC peaks.

*2.9.6. Fourier transform infrared analysis (FTIR)*

The IR spectrum of fatty acids and FAMEs of diatom oil was recorded in FTIR spectrometer (PerkinElmer GX FTIR) to identify the functional groups corresponding to lipids by scanning the liquid sample (containing fatty acids/FAMEs extracted in hexane) at mid infra-red region (4000–650 $cm^{-1}$) under transmission mode. The absorption spectra of fatty acids and FAMEs were plotted between % transmittance versus wave number.

## 3. Results and discussions

### 3.1. Screening and identification of lipase producing fungal strains

Eight strains (CS1 – CS8) exhibited positive characteristics to extracellular lipase secretion on agar medium based lipase screening tests among the twenty-four fungal strains (endophytic/fee-spores) isolated from the high saline region,. These eight strains were further scaled down to one strain (CS4) based on the observation of distinctively higher zone of clearance, usually measured by the degradation halo of enzyme-specific substrates. Morphological examination of conidia and conidiophores under high-resolution optical microscope after lactophenol cotton blue staining revealed the pattern of conidial spores belonging to the genera *Cladosporium* sp. SEM visualization of the grown hyphae and mycelia reconfirmed the identification of filamentous fungus to be *Cladosporium* sp. The light microscopy and SEM images of the *Cladosporium* sp. is presented in Fig. 3. The results of tween80 - $CaCl_2$ and olive oil - phenol red-based lipase screening test

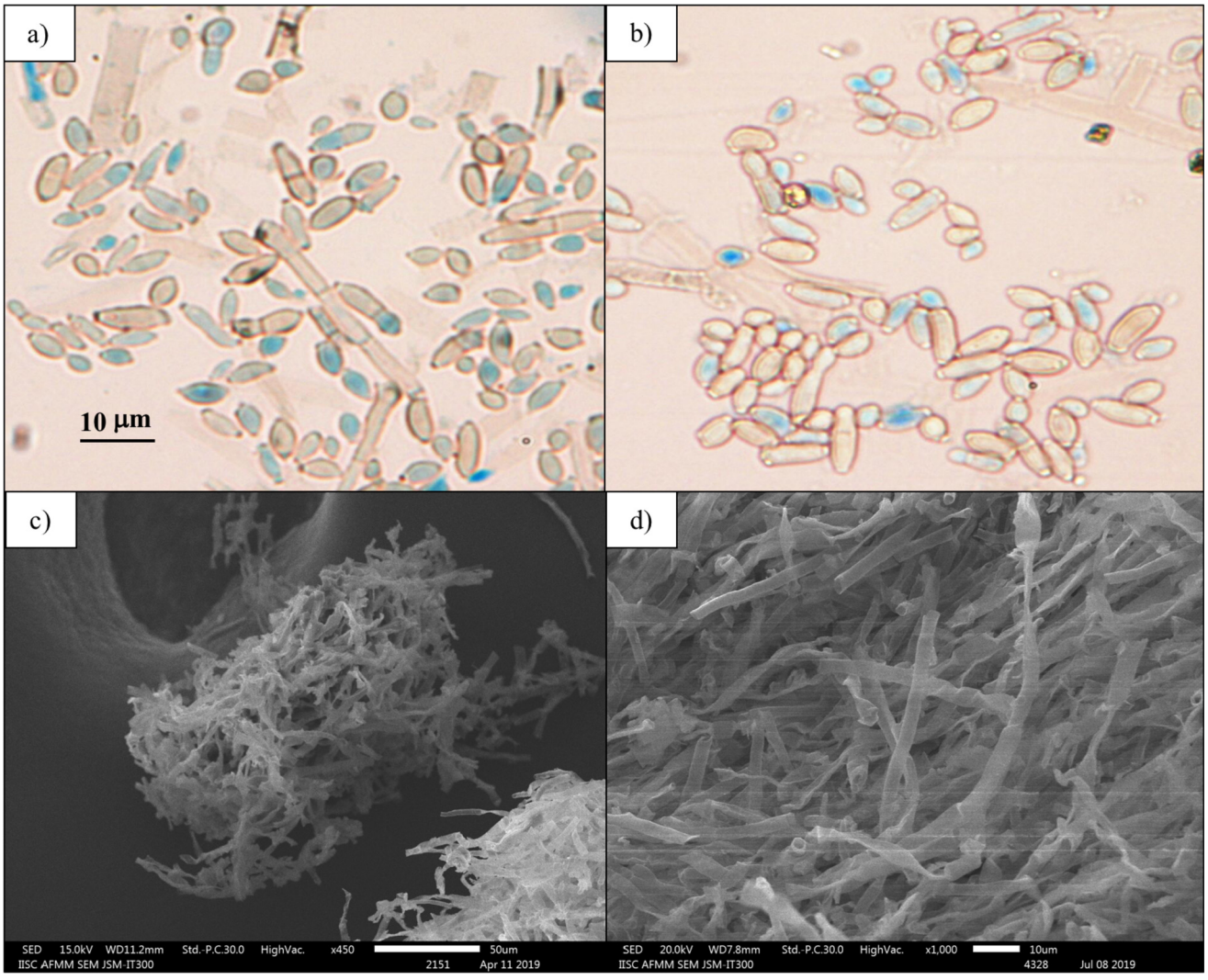


**Fig. 3.** Light microscopy and SEM images of *Cladosporium* sp. CS4.

displaying zones of clearance are presented in Fig. 4. The molecular identification and phylogenetic analysis of the strain CS4 revealed maximum homology with the fungus *Cladosporium tenuissimum*, identified hereafter as *Cladosporium* sp. CS4 is presented in the form of a phylogenetic tree in Fig. 5. The prospects of lipase extracted from this genera as a potential biocatalyst for biodiesel production is reported for the first time through this work though there are some reports of *Cladosporium* sp. extracted lipase [22,62].

Two different strains of *Cladosporium cladosporioides* were earlier [62] isolated and reported from rotten maize for determining variations in lipase enzyme activity, revealed exceedingly higher levels of lipase activity on agar plates evident from the higher zone of clearance among a total of twenty-three other fungal isolates. Moreover, the lipolytic activity exhibited by *C. Cladosporioides* was much higher than that of *P. solitum* [63].

### 3.2. Enzyme production, purification and molecular weight determination

The ambient conditions of all enzyme purification steps were maintained at 4 °C. *Cladosporium* sp. CS4 produced extracellular crude lipase was subjected to partial purification/protein concentration using ammonium sulphate precipitation. The pellet obtained after ammonium sulphate precipitation was suspended in 0.5 M Tris-Chloride buffer at pH 7.2 and dialyzed using 3.5 KDa Molecular Weight Cut off (MWCO) against the same buffer with 0.1 M NaCl. Gel filtration chromatography was performed by elution of partially purified enzyme using buffers containing appropriate salt concentrations after proper degassing of buffers and deionized water used for equilibrating the column. Gel filtration-based size-exclusion chromatographic system uses a hydrophilic packing material and an aqueous mobile phase to fractionate water soluble proteins based on their differences in its molecular weight. When dissolved protein molecules of various sizes flows through the column, larger proteins elute first as the pores of the hydrophilic gel packed inside the column is too small for the larger proteins to enter, whereas smaller proteins elute slower after entering the pores of the packed gel column thus leading to effective sorting and separation of proteins based on size [64]. Superdex 200 column filtration of the enzyme resulted in a purification fold of 4.1 with an activity recovery (yield) of about 36.7% and specific activity of about 37.2 U/mg. Table 1 lists the gradation in improved activity, purity and yield at each stage of enzyme purification. The present study show comparable yield

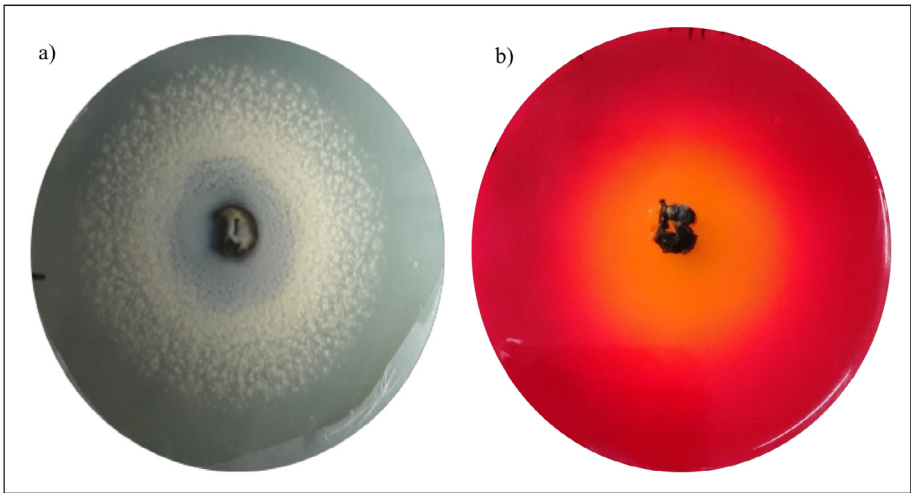


**Fig. 4.** Degradation halo zones exhibited by *Cladosporium* sp. CS4 in a) $CaCl_2$-tween 80 and b) phenol red-olive oil agar plates.

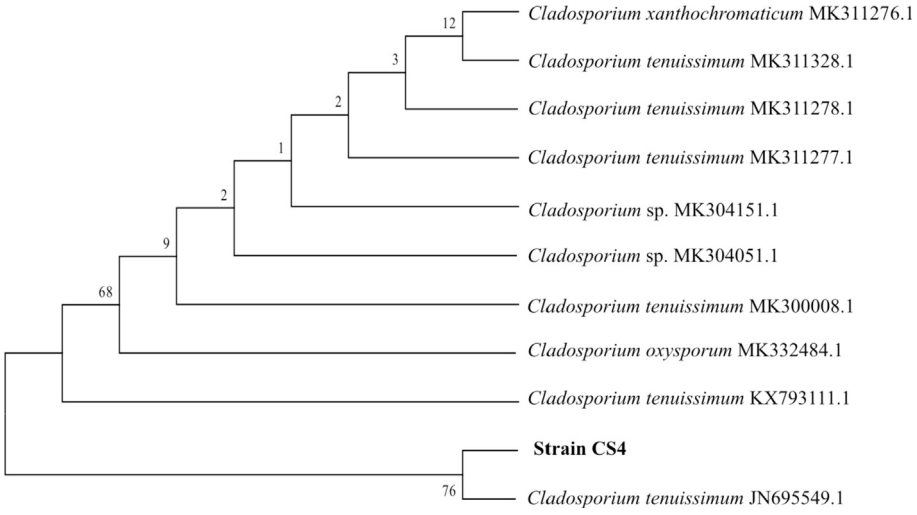


**Fig. 5.** Phylogenetic analysis exhibiting homology of strain CS4 with *Cladosporium tenuissimum*.

**Table 1**
Summary indicating improvement in purification after each enzyme purification step.

| Diatom | Lipid Content (%) dcw | Reference |
|---|---|---|
| *Achnanthes delicatulum hauckiana* | 29.8 | [105] |
| *Achnanthes* sp. | 27.7 | [106,107] |
| *Aulacoseira Ambigua* | 19.7 | [108] |
| *Bacillaria paradoxa* | 33.61 | [109,110] |
| *Cocconeis peltoides* | 20.9 | [105] |
| *Chaetoceros curvisetus* | 14.86 | [111] |
| *Diatoma* sp. | 15.76 | [105,112] |
| *Cocconeis* sp. | 31.81 | [113] |
| *Cyclotella cryptica* | 27 | [114] |
| *Melosira* sp. | 14.75 | [115] |
| *Nitzschia dissipata* var. *media* | 37.5 | [114,116] |
| *Phaeodactylum tricornutum* | 10.7 | [117] |
| *Synedra ulna* | 7.58 | [55] |
| *Tryblionella navicularis* | 24.2 | [105] |
| *Nitzschia punctata* | 16 | This study |

with higher purification than reported earlier [33]. Lipase extraction and characterization on a yeast strain *Ralstonia* sp. resulted in 3.9-fold purity and 20.8% yield respectively [33]. Lipase purification on a biocontrol fungus *Nomuraea rileyi* exhibited a yield of 1.69% with a purification fold of 23.9 [65]. Fungi *Penicillium expansum* had reported the highest purification fold of 219.0, with a poor yield of 5% [66]. Purification and characterization of lipase extracted from a bacterium *Pseudomonas aeruginosa* reported 8.6-fold purification with 51.6% activity recovery [51]. After purification, aliquots of 40 μL crude culture filtrate, partially purified and purified protein was loaded onto SDS PAGE with 10% polyacrylamide gel to observe visible bands of lipase as against protein molecular markers. The molecular weight of the purified protein was ~46 KDa through SDS PAGE. SDS PAGE gel representing different lanes with enzyme and protein molecular marker is given in Fig. 6.

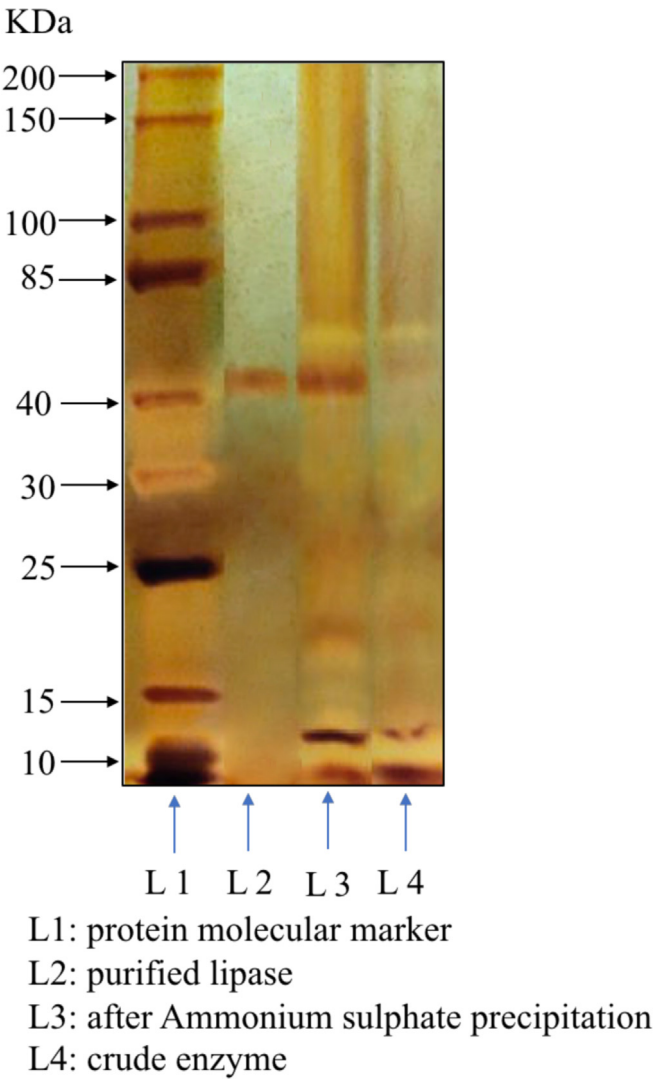


**Fig. 6.** Characterization of enzyme at each stages of purification.

### 3.3. Enzyme characterization

#### 3.3.1. Effects of pH and temperature

The structural and functional relationships between enzymes and reaction media are primarily determined by ambient environmental conditions, especially pH and temperature [67]. Fig. 7 represents varying temperature and pH versus relative and residual enzyme activity. The lipase activity of the enzyme was maximum at pH 6 and at a temperature of 60 °C. The relative enzyme activities at pH 4.0 and pH 5.0 were 34 ± 7% and 31 ± 8% to that of the maximal lipase activity observed at pH 6.0. Significantly higher lipase activity was recorded at pH 6.0, with <35% of the relative activities observed at other pH ranges depicting the sensitiveness of lipase to varying pH ranges. Mycelium bound intracellular lipase extracted from the fungus *Aspergillus niger* showed optimal pH in the acidic range from 3.0 to 6.5 with higher activity at the pH of 4.0 [68]. *Candida cylindracea* yeast extracted lipase [69] showed maximum lipase activity at pH 6.0. However, intracellular lipase extracted from fungi *Aspergillus westerdijkiae* [70], *Nomuraea rileyi* [42] and *Rhizopus oryzae* [71] exhibited optimal pH activity on the alkaline pH (8.0–8.5). The optimum temperature at which the lipase enzyme showed maximum activity was 60 °C and fairly high stabilities were recorded at higher temperatures of 40 °C (95 ± 1%) and 50 °C (99 ± 1.5%), which are comparable to the performance of *Mrakia blollopis* yeast extracted lipase [49].

#### 3.3.2. Effect of surfactants on lipase activity

Long-chain ionic liquids like surfactants have a tendency to alter its physicochemical properties by internal alteration of charges from cationic to anionic and vice versa [72]. These surfactants aid in providing enzymes with access to substrates through interfacial area stabilization, which enhanced the catalytic reaction of lipase [50]. Surfactants are important components of emulsion preparations during lipase assays at every stage from enzyme production, purification and characterization [73]. Fig. 8 illustrates the effect of different surfactants on lipase enzyme activity. Lipase enzyme showed variation in affinities to each substrate, when introduced into different concentrations of surfactants like Triton X100, SDS, Tween 80 and Tween 20. Triton X100 had less affinity to enzymes with least activity exhibited among other surfactants. The addition of varying percentage concentrations of SDS increased the lipase activity with maximum activity recorded at SDS concentration of 0.25% with 4.02 ± 0.27 U/mL. Higher concentrations of SDS was found to inhibit the enzyme activity. Enzyme activity was less yet uniform among different concentration ranges of tween 80, however, for tween 20, activity for a concentration of 0.5% (2.89 ± 0.61 U/mL) was found to be the maximum. Fungus *Nomuraea rileyi* showed enhanced lipolytic activities in the presence of SDS and tween 80 which is similar to the results of the present study [73], whereas lipase extracted from thermophilic *Rhizopus oryzae* and a yeast *Yarrowia lipolytica* showed decreased with inhibited activities when introduced to SDS and tween 80 [74,75].

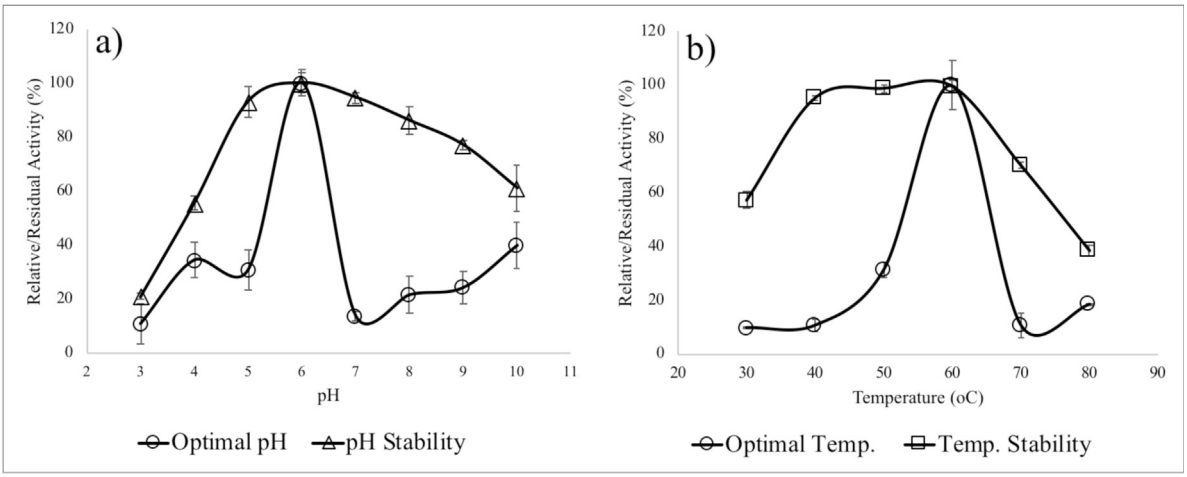


**Fig. 7.** a) Effect of pH on lipase activity and pH stability of lipase b) Effect of temperature of lipase activity and residual activity at different temperatures.

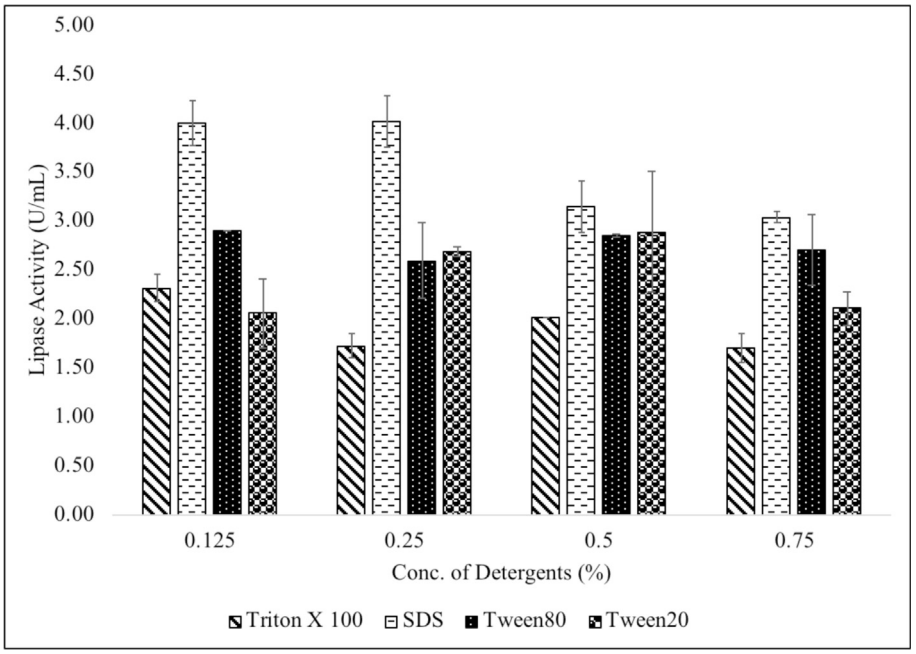


**Fig. 8.** Effect of different surfactants and its concentration on lipase enzyme activity.

### 3.4. Isolation, growth and SEM analysis of microalgae

Initial agar plating (spread plate method) and subculturing by quadrant streaking of epipelic diatoms (diatoms attached to sediments) collected from the salt pan, resulted in the isolation of pure diatoms. Morphological characterization of the diatom isolates using high-resolution phase contrast optical microscope and SEM visualization aided in species-level identification of the diatom. Comparison of high-resolution images with standard identification keys confirmed the diatom as *Nitzschia punctata.* It is a pennate diatom with valves elliptic-lanceolate and apices rostrate, striae more or less curved, with transverse, radiant and moniliform forming coarse puncta placed at equal distances [53,76]. Apices have no nodules with often showing fine scattered puncta. The alga measures ~13. 5–14. 5 μm in length and ~5.6–6.0 μm in width. The light microscopy images of *Nitzschia* sp. in a colony with evident extracellular polymeric secretion (EPS) matrix in the background and SEM images of the culture is presented in Fig. 9.

### 3.5. Algal biomass concentration and productivity determination

Diatoms grown in 10-L culture tubs at the rooftop in the natural daylight were harvested after nine days of cultivation period. The algal biomass was harvested through simple siphoning of spent culture medium and washed thrice using deionized water to ensure the complete removal of traces of salt from algal biomass and subsequently, oven-dried at 85 °C overnight. The dried diatom biomass exhibited a biomass concentration of 354 ± 0.24 mg $L^{-1}$ and biomass productivity of 39.3 ± 0.14 mg $L^{-1}$ $d^{-1}$. Benthic diatom *Navicula cincta* [77] reported a biomass concentration of 318.5 mg $L^{-1}$ [78] and diatom *Cylindrotheca closterium* resulted in biomass production of 356 mg $L^{-1}$. Similar work [79] that compared growth rate and biomass productivities of twenty-two different genus of microalgae reported the biomass concentrations and

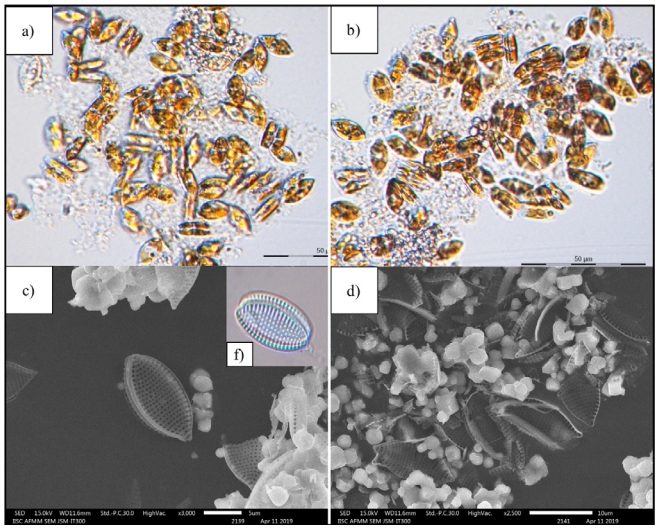


**Fig. 9.** a, b) Light microscope images of live *Nitzschia punctata* culture with a matrix of EPS at the background c, d) SEM images of processed *Nitzschia* culture f) acid digested silica frustules of *Nitzschia* sp. imaged under 100x oil immersion in phase contrast optical microscope.

productivities of two different strains of *Cylindrotheca fusiformis* to be 357 and 366 mg $L^{-1}$ and 55 and 52 mg $L^{-1}$ $d^{-1}$ respectively. The biomass concentration and productivity values reported in other studies are very much comparable to the present result. Only a hand few studies that concentrate on biodiesel prospects of diatoms among other microalgae [55,78–82]. *Nitzschia* sp. was explored for its potential as a third-generation feedstock for biodiesel production is reported for the first time.

### 3.6. Lipid extraction and quantification

In-situ environmental parameters during diatom growth act as a decisive factor in the accumulation of lipids and the lipid content [83]. Generally, nitrate and silicate nutrient deprivation in diatoms has induced higher lipids [84]. The quantity of lipids present in *Nitzschia* sp. was gravimetrically determined after lipid extraction using Soxhlet apparatus. The culture showed 16% lipid content with an estimated oil yield 16% of the algal biomass used for lipid extraction based on dry cell weight after nine days of outdoor cultivation under sunlight. As the diatoms were grown under nutrient replete conditions, the lipid content is comparatively lower than the values reported in other studies on diatoms. Table 2

**Table 2**
Lipid content across different diatom species.

| Diatom | Lipid Content (%) dcw | Reference |
|---|---|---|
| *Achnanthes delicatulum hauckiana* | 29.8 | [105] |
| *Achnanthes* sp. | 27.7 | [106,107] |
| *Aulacoseira Ambigua* | 19.7 | [108] |
| *Bacillaria paradoxa* | 33.61 | [109,110] |
| *Cocconeis peltoides* | 20.9 | [105] |
| *Chaetoceros curvisetus* | 14.86 | [111] |
| *Diatoma* sp. | 15.76 | [105,112] |
| *Cocconeis* sp. | 31.81 | [113] |
| *Cyclotella cryptica* | 27 | [114] |
| *Melosira* sp. | 14.75 | [115] |
| *Nitzschia dissipata* var. *media* | 37.5 | [114,116] |
| *Phaeodactylum tricornutum* | 10.7 | [117] |
| *Synedra ulna* | 7.58 | [55] |
| *Tryblionella navicularis* | 24.2 | [105] |
| *Nitzschia punctata* | 16 | This study |

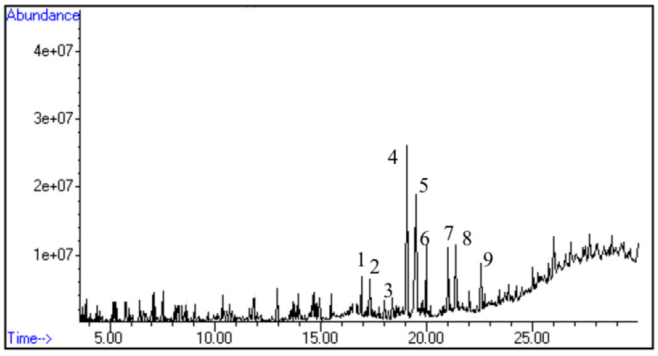


**Fig. 10.** Fatty acid profile of lipids extracted from *Nitzschia* sp.

lists the estimated lipid contents of different genera of a pennate diatom.

### 3.7. Fatty acid characterization

Fatty acid profiles of biofuel feedstock help in assessing biodiesel quality [85]. Biodiesel quality is significantly governed by the nature of fatty acids in terms of percentage composition, degree of saturation/unsaturation and the length of the carbon chains present [86,87]. Fig. 10 illustrates the peaks of fatty acids extracted from *Nitzschia* sp. Fatty acid profiling of lipids extracted from *Nitzschia punctata* resulted in higher proportions of C16 and C18 fatty acids (Table 3). GC-MS peaks recorded the presence of nine different fatty acids, with a predominance of saturated fatty acids. Long-chain fatty acids such as linoleic acid, trans-vaccenic acid and arachidic acid were effectively converted into methyl esters by lipase biocatalyst extracted from Cladosporium sp. CS4, which highlights selective and highly specific enzymatic reactions on microalgal oil. Similar fatty acids profile was reported earlier in marine microalgae *Tetraselmis* sp. [88].

### 3.8. FAME composition analysis and biodiesel yield estimation

The compositional distribution of FAMEs is listed in Table 4. Fig. 11 highlights GC-MS peaks representing different FAMEs eluted in acid-catalyzed (ACT) and lipase-catalyzed (LCT) treatments of diatom oil. Ten different FAME mixtures were observed in GCMS peaks of acid-catalyzed and enzyme-catalyzed biodiesel. FAMEs showed a dominance of saturated fatty acids (SFAs) with larger proportions of C16:0 (palmitic acid methyl esters) followed by monounsaturated fatty acid (MUFAs) C18:1 (oleic acid methyl esters). An increase in saturated and monounsaturated fatty acids with an increase in salinity in *Dunaliella* sp. was reported with a significant decrease in polyunsaturated fatty acids (PUFAs). methyl esters of palmitoleic acid (C16:1), trans- Vaccenic acid (18:1)

**Table 3**
Fatty acid composition of diatom oil.

| Peak No. | RT (min.) | Fatty acids | Peak area | Peak area (%) |
|---|---|---|---|---|
| 1 | 16.61 | Myristic acid | 40767584 | 1.10 |
| 2 | 16.89 | Pentadecanoic acid | 81024700 | 2.19 |
| 3 | 17.99 | Palmitoleic acid | 152336989 | 4.13 |
| 4 | 19.05 | Palmitic acid | 1774512688 | 48.17 |
| 5 | 19.49 | Linoleic acid | 751427658 | 20.40 |
| 6 | 20.89 | Oleic acid | 462672390 | 12.56 |
| 7 | 21.00 | Trans - Vaccenic acid | 225839717 | 6.13 |
| 8 | 21.35 | Stearic acid | 130207782 | 3.53 |
| 9 | 22.58 | Arachidic acid | 64554901 | 1.75 |

**Table 4**
Compositional variation of acid catalyzed and enzyme catalyzed microalgal (diatom) biodiesel.

| Peak No. | RT (min.) | FAMEs | Carbon Chain Length | LCT | | ACT | |
|---|---|---|---|---|---|---|---|
| | | | | Peak Area | % out of total | Peak Area | % out of total |
| 1 | 16.88 | Myristic acid, methyl ester | C14:0 | 108676936 | 4.10 | 4823046 | 5.95 |
| 2 | 17.98 | Pentadecanoic acid, methyl ester | C15:0 | 4304883 | 0.12 | 2665956 | 3.28 |
| 3 | 18.82 | Palmitoleic acid, methyl ester | C16:1 | 7509694 | 0.28 | – | – |
| 4 | 19.08 | Palmitic acid, methyl ester | C16:0 | 1407095829 | 53.12 | 44784447 | 55.22 |
| 6 | 20.70 | Linoleic acid, methyl ester | C18:2 (n-3) | 292828011 | 11.05 | – | – |
| 7 | 20.78 | Oleic acid, methyl ester | C18:1 | 642377234 | 24.25 | 12412166 | 15.30 |
| 8 | 20.81 | trans-Vaccenic acid, methyl ester | C18:1 | 37348396 | 1.41 | – | – |
| 9 | 20.99 | Stearic acid, methyl ester | C18:0 | 145464005 | 5.49 | 2975013 | 3.67 |
| 10 | 21.99 | Octadecanoic acid, 9,10,12-trimethoxy-methyl ester | C18:3 | – | – | 7178879 | 8.85 |
| 11 | 22.54 | Octadecanoic acid, 9,10-dichloro-, methyl ester | C18:2 | – | – | 6252417 | 7.71 |
| 12 | 22.78 | Arachidic acid | C20:0 | 2848166 | 0.102 | – | – |

*LCT – Lipase catalyzed transesterification.
ACT – Acid catalyzed transesterification.

linoleic acid (C18:2) n-3 which were absent in acid-catalyzed biodiesel sample made its presence in lipase-catalyzed biodiesel [89]. The similar appearance of methyl esters of monounsaturated fatty acids was found in NaCl treated cells of *Chlorella* sp. to that of control without NaCl was reported by Ref. [90]. In enzyme-catalyzed biodiesel, mix of SFAs and MUFAs - palmitic (53.12%), oleic (24.25%) and linoleic (11.05%) acid methyl esters constituted up to 88.4% of the total fatty acids present in the sample, whereas in acid-catalyzed biodiesel mixes of SFAs and PUFAs - palmitic (55.22%), myristic (5.95%) and pentadecanoic acid (3.28%) and octadecanoic acids (16.56%) constituted up to 84.65%. Algal oil with a higher composition of PUFAs is inappropriate as fuel for engines as PUFAs lead to lower oxidative stability of the fuel which in turn affects engine performance [91].

The qualitative analysis of *Nitzschia* sp. generated FAMEs through lipase-catalyzed transesterification showed a predominance of saturated (62.83%) and MUFA (37.0%), thus possessing the characteristics to produce good quality biodiesel with higher oxidative stability. On the other hand, in acid-catalyzed biodiesel C16:0 and C18:1 were predominant however, MUFAs palmitoleic, linoleic and trans vaccenic acid were absent. An ideal mix of C16:1, C18:1 and C14:0 in the mass ratio of 5:4:1 produce superior quality biodiesel with higher cetane number (CN), good cold filter plug flow properties (CFPP) and better oxidative stability [92,93]. FAMEs generated by lipase-catalyzed transesterification has not yielded C16:1, C18:1 and C14:0 at exact proportions, but GC-MS shows the presence of all three important FAMEs. Tweaking environmental parameters would result in good quality biodiesel with prescribed fatty acid composition [92]. Biodiesel yield calculations were carried out on LCT and ACT samples using [13] highlight higher FAME yield of 87.24% for lipase-catalyzed transesterification when compared to acid-catalyzed transesterification yield of 83.08%. Thus, FAME yield confirms the superior performance of biocatalyst over conventional chemical catalysts in the transformation of microalgal oil into biodiesel.

### 3.9. FT-IR analysis of algal oil and biodiesel

FT-IR spectrum of algal oil and biodiesel derived from algal oil by acid and enzyme-catalyzed transesterification is given in Fig. 12a–d. Fig. 12a represents the IR spectrum of hexane extracted algal fatty acids, while Fig. 12b and c represents FAMEs extracted from algal oil using acid (ACT) and enzyme (LCT) catalysts. Fig. 12d represents the comparative graph of all three FT-IR peaks together. FT-IR bands at 3009.5 $cm^{-1}$, 2854.08 $cm^{-1}$ indicates the presence of C – H stretching vibrations of alkanes while the peak of 1465.28 $cm^{-1}$ indicates bending vibrations of C – H alkane bonds (methylene group) [94] reflected from hexane as the fatty acids and FAMEs are extracted in hexane. The peak at 1379.5 $cm^{-1}$ is due to symmetric bending of $\delta_s$ (CH) $CH_3$ vibrational modes, a probable 1-hexene derivative of hexane. The weak band at 1238.3 $cm^{-1}$ is due to strong stretching vibrations of (C – O) alkyl aryl ether [95]. The strong peak at 1745.44 $cm^{-1}$ represents C = O stretching of esters in methoxy carboxylic acids confirms the presence of higher proportions of free fatty acids and FAMEs [96,97]. The peak at 1465.28 $cm^{-1}$ represents the medium C – H bending stretch of alkane (methylene) group. The peak 1163.15 $cm^{-1}$ represent stretching vibration of C – O – C ester (formates) groups. The vibration at 722.56 $cm^{-1}$ is due to bending vibrations of = C – H of alkenes and aromatics [98]. Extra peaks at 1157 $cm^{-1}$, 955.9 $cm^{-1}$ and 724 $cm^{-1}$ in the lipase-catalyzed biodiesel, indicates strong C – O stretching of aliphatic ether and C = C bending of alkene compounds respectively. The FT-IR highlights the predominance of C – H group confirming the suitability of algal oil as biodiesel [99], similar to the earlier studies [60,96,100] for vegetable oil as

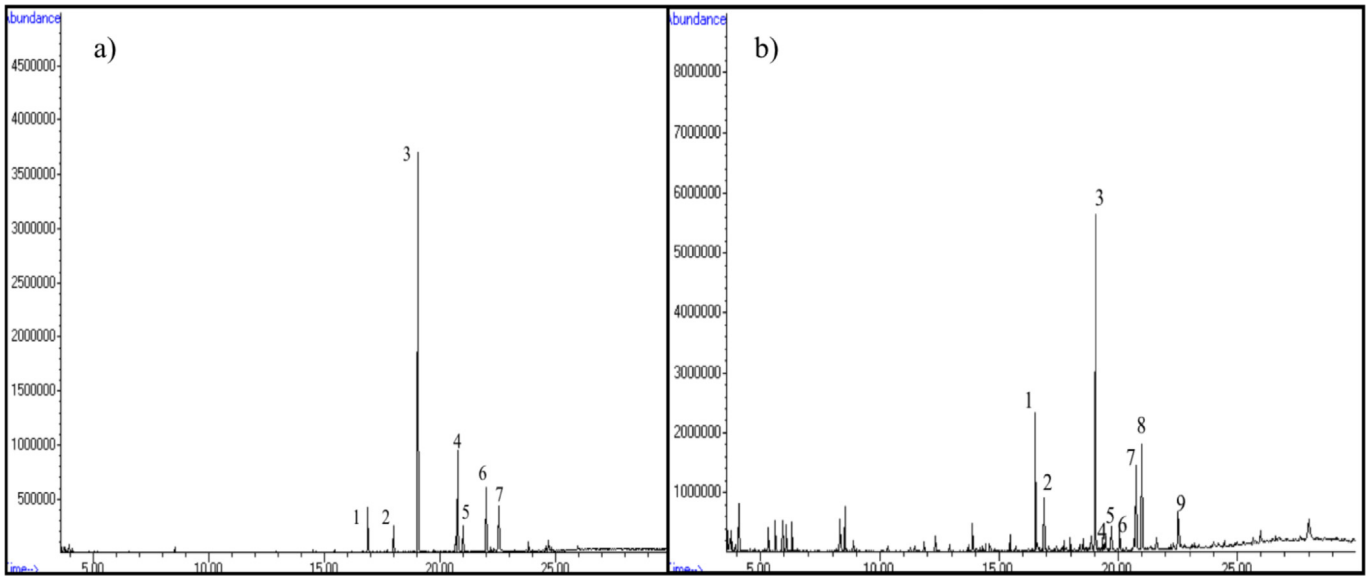


**Fig. 11.** FAME composition of a) ACT and b) LCT diatom biodiesel.

well as algal oil-derived biodiesel.

The analyses confirm the prospects of using fungal lipase as biocatalyst for effective conversion of algal oil to biodiesel. The results indicate the suitability of *Cladosporium tenuissimum* extracted lipase as a low-cost biocatalyst over conventional acid catalysts with assured environmentally-friendly bioproducts. Optimization of reaction conditions such as reaction temperature, agitation speed, the volume of methanol and enzyme for enzyme-catalyzed transesterification through design of experiments would lead to enhanced FAME yields. Moreover, experiments utilizing *Cladosporium tenuissimum* as a whole-cell biocatalyst in free or immobilized form would open new avenues of sustainable biodiesel production through a scope for enzyme reuse, which reduces production costs.

### 3.10. Comparative assessment of biodiesel yield

Lipase catalyzed transesterification of microalgal oil is usually

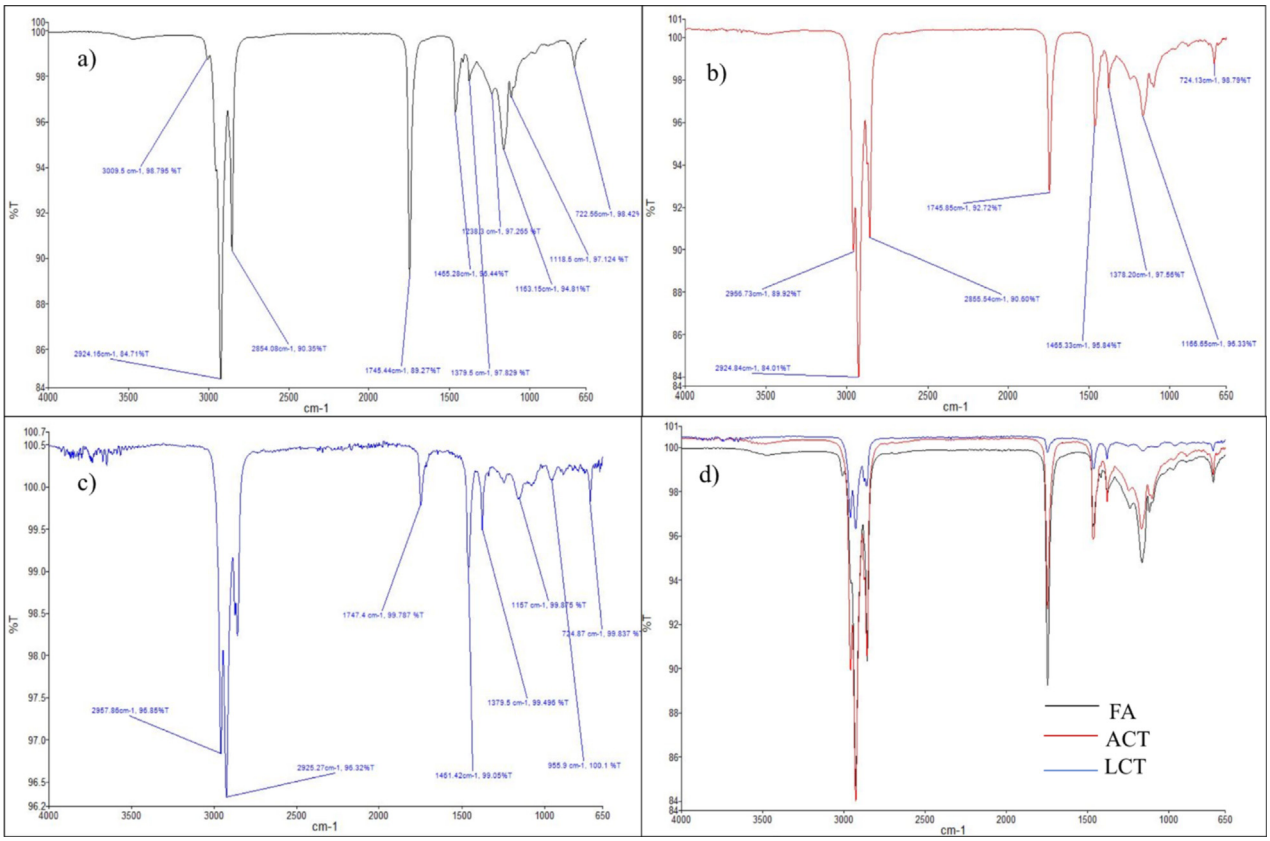


**Fig. 12.** FT −IR spectra of fatty acid and FAME extracted from *Nitzschia* sp.

**Table 5**
Comparison of FAME yields in lipase catalyzed transesterification of different generations' biodiesel feedstocks.

| Feedstock used | Lipase extraction source (organisms) | Type/Nature of Lipase | FAME yield (%) | References |
|---|---|---|---|---|
| Sardine oil | *Rhizopus oryzae* | Immobilized | 76.5 | [74] |
| Sardine oil | *Mucor hiemalis* | immobilized | 38.4 | [118] |
| Sardine oil | *Candida rugosa* | immobilized | 50 | [119] |
| Soybean oil | *Pseudomonas cepacia* | Gel entrapment | 67 | [120] |
| Jatropha oil | *Chromobacterium viscosum* | Celite 545 entrapment | 71 | [121] |
| Waste oil, plant oil | *Candida* sp. | Cotton membrane immobilization | 90–92 | [122] |
| *Chlorella vulgaris ESP 31* | *Burkholderia* sp. *C20* | immobilized | 92.15–95.75 | [123] |
| *Gracilaria edulis* | *Candida antartica lipase in Pichia pastoris (Cal A, Cal B)* | Free, Immobilized | 88.5–93 | [59] |
| *Enteromorpha compressa* | *Candida antartica lipase in Pichia pastoris (Cal A, Cal B)* | Free, Immobilized | 89–92 | [59] |
| *Ulva lactuca* | *Candida antartica lipase in Pichia pastoris (Cal A, Cal B)* | Free, Immobilized | 87–90 | [59] |
| *Scenedesmus obliquus* | *Aspergillus niger* | Immobilized in BSPs | 90.82 | [37] |
| *Chlorella salina* | *Rhodotorula mucilaginosa* | Immobilized | 85.29 | [34] |
| *Chlorella* sp. | *Mold-fungus JN7* | Immobilized in BSPs | 50.3 | [124] |
| *Marine microalgae DY54* | *Mold fungus JN7* | Immobilized in BSPs | 68.2 | [124] |
| *Chlorella protothecoides* | *Candida antarctica (type B)* | Free and Immobilized in combination | 97 | [36] |
| *Tetraselmis* sp. | *Candida rugosa* | Commercial | 7 folds higher FAME conversion rate | [20] |
| *Nitzschia punctata* | *Cladosporium tenuissimum* | Free | 87.2 | This study |

carried out in two different ways: i) using lipase enzyme either extracted from extracellular or intracellular enzyme secretions of fungi, yeast or bacteria as a source of catalyst and (ii) Using fungal/bacterial (immobilized) cell as a whole cell lipase. *Microbacterium* sp. [101], *Pseudomonas* sp [102,103]. are the bacterium explored for its lipase and *Aspergillus* sp. [37], and *Candida* sp [104]. are the fungal and yeast species explored earlier. However, *Cladosporium* sp. extracted lipase used for the production of microalgal biodiesel is a first of its kind study with prospective scope for future research. Earlier, *Chlorella* sp. and *Scenedesmus* sp. were the widely studied microalgal species for lipase mediated transesterification with reported FAME yields ranging between 85 and 97% [37,41,102]. The analyses of diatom lipids for the production of biodiesel using lipase provide important insights on the potential of diatoms to be explored for biodiesel prospects. Comparison of FAME yields in lipase catalyzed biodiesel production from available literature with that of the current study is listed in Table 5.

## 4. Conclusion

The *Cladosporium tenuissimum* CS4 fungal strain extracted lipase has proven characteristics to be a low-cost biocatalyst over conventional acid catalysts for microalgal biodiesel production with assured environmentally friendly bioproducts. *Cladosporium* sp. extracted lipase being used as a biocatalyst for optimizing microalgal biodiesel production is reported for the first time in this study. Biochemical and enzyme characterization of such thermotolerant enzyme could aid as an industrially important enzyme serving diverse interdisciplinary applications. The extracted and purified enzyme is effective in catalyzing the conversion of microalgal oil into biodiesel with higher efficiency (87.2 ± 0.47%) compared to the conventional acid catalyst (83.02 ± 0.35%). Future studies on protein characterization, sequencing and enzyme immobilization using different solid substrate sources or chemical methods are expected to improve the enzyme viability and reuse.

## Data and accessibility

Data used in the analyses are compiled from the field. Data is anlysed and organized in the form of table, which are presented in the manuscript. Also, synthesized data are archived at
http://wgbis.ces.iisc.ernet.in/energy/paper/researchpaper.html#bio.
http://wgbis.ces.iisc.ernet.in/biodiversity/

## Funding

The authors acknowledge the financial support from ISRO – IISc space technology cell [ISTC/BES/TVR/389] and Gajanana and Madhavi Hegde [CES/TVR/GJH001] endowment for biofuel research.

## Research ethics

The publication is based on the original research and has not been submitted elsewhere for publication or web hosting.

## Animal ethics

The research does not involve either humans, animals or tissues.

## Permission to carry out fieldwork

Ecological research carried by us in the Western Ghats is commissioned by the Ministry of Environment, Forests and Climate Change (ENVIS Division), Government of India and hence no further permission is required as the field work was carried out in the non-restricted areas/protected areas.

## Declaration of competing interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

## CRediT authorship contribution statement

**G. Saranya:** Data curation, Writing - original draft. **T.V. Ramachandra:** Formal analysis, Writing - review & editing.

## Acknowledgements

The authors acknowledge the financial support from ISRO – IISc space technology cell [ISTC/BES/TVR/389] and Gajanana and Madhavi Hegde [CES/TVR/GJH001] endowment for biofuel research. We are grateful to the researchers at Prof. D. N Rao's lab, Department of Biochemistry, Indian Institute of Science for providing the infrastructure support, guidance and useful inputs for enzyme characterization and purification. We thank central facilities of IISc – Society for Innovation and Development for Mass spectroscopy (GC-MS) analysis, Advanced Facility for Microscopy and

Microanalysis for SEM imaging and Micro Nano characterization Facility (MNCF) for FT-IR analysis.

## References


[1] T.V. Ramachandra, R. Jain, G. Krishnadas, Hotspots of solar potential in India, Renew. Sustain. Energy Rev. 15 (2011) 3178–3186, https://doi.org/10.1016/J.RSER.2011.04.007.

[2] E.M. Shahid, Y. Jamal, Production of biodiesel: a technical review, Renew. Sustain. Energy Rev. 15 (2011) 4732–4745, https://doi.org/10.1016/J.RSER.2011.07.079.

[3] T.V. Ramachandra, M. Durga Madhab, S. Shilpi, N.V. Joshi, Algal biofuel from urban wastewater in India: scope and challenges, Renew. Sustain. Energy Rev. 21 (2013) 767–777, https://doi.org/10.1016/j.rser.2012.12.029.

[4] A.E. Atabani, A.S. Silitonga, I.A. Badruddin, T.M.I. Mahlia, H.H. Masjuki, S. Mekhilef, A comprehensive review on biodiesel as an alternative energy resource and its characteristics, Renew. Sustain. Energy Rev. 16 (2012) 2070–2093, https://doi.org/10.1016/J.RSER.2012.01.003.

[5] Y. Su, K. Song, P. Zhang, Y. Su, J. Cheng, X. Chen, Progress of microalgae biofuel's commercialization, Renew. Sustain. Energy Rev. 74 (2017) 402–411, https://doi.org/10.1016/j.rser.2016.12.078.

[6] X.-G. Zhu, S.P. Long, D.R. Ort, What is the maximum efficiency with which photosynthesis can convert solar energy into biomass? Curr. Opin. Biotechnol. 19 (2008) 153–159, https://doi.org/10.1016/J.COPBIO.2008.02.004.

[7] J.S. Boyer, Plant productivity and environment, Science 218 (1982) 443–448, https://doi.org/10.1126/science.218.4571.443.

[8] Y. Guo, T. Yeh, W. Song, D. Xu, S. Wang, A review of bio-oil production from hydrothermal liquefaction of algae, Renew. Sustain. Energy Rev. 48 (2015) 776–790, https://doi.org/10.1016/J.RSER.2015.04.049.

[9] A. Darzins, P.T. Pienkos, The Promise and Challenges of Microalgal-Derived Biofuels, 2009, https://doi.org/10.1002/bbb.159.

[10] Y. Chisti, M. Seibert, R. Davis, N. Sweeney, S. Wychen, A. Lowell, P. Pienkos, I. Archibald, J. Granados, C. Greene, Biodiesel from microalgae, Biotechnol. Adv. 25 (2007) 294–306, https://doi.org/10.1016/j.biotechadv.2007.02.001.

[11] F. Moazeni, Y.-C. Chen, G. Zhang, Enzymatic transesterification for biodiesel production from used cooking oil, a review, J. Clean. Prod. 216 (2019) 117–128, https://doi.org/10.1016/J.JCLEPRO.2019.01.181.

[12] B. Kayode, A. Hart, An overview of transesterification methods for producing biodiesel from waste vegetable oils, Biofuels (2017) 1–19, https://doi.org/10.1080/17597269.2017.1306683.

[13] Z. Amini, H.C. Ong, M.D. Harrison, F. Kusumo, H. Mazaheri, Z. Ilham, Biodiesel production by lipase-catalyzed transesterification of Ocimum basilicum L. (sweet basil) seed oil, Energy Convers. Manag. 132 (2017) 82–90, https://doi.org/10.1016/J.ENCONMAN.2016.11.017.

[14] A. Louwrier, Industrial products - the return to carbohydrate-based industries, Biotechnol. Appl. Biochem. 27 (1998) 1–8, https://doi.org/10.1111/j.1470-8744.1998.tb01368.x.

[15] F. Beisson, V. Arondel, R. Verger, Assaying Arabidopsis lipase activity, Biochem. Soc. Trans. 28 (2000) 773–775, https://doi.org/10.1042/0300-5127:0280773.

[16] M.I. Van Dyke, H. Lee, J.T. Trevors, Applications of microbial surfactants, Biotechnol. Adv. 9 (1991) 241–252, https://doi.org/10.1016/0734-9750(91)90006-H.

[17] M.-L. Jia, X.-L. Zhong, Z.-W. Lin, B.-X. Dong, G. Li, Expression and characterization of an esterase belonging to a new family via isolation from a metagenomic library of paper mill sludge, Int. J. Biol. Macromol. 126 (2019) 1192–1200, https://doi.org/10.1016/J.IJBIOMAC.2019.01.025.

[18] B. Ozcan, G. Ozyilmaz, C. Cokmus, M. Caliskan, Characterization of extracellular esterase and lipase activities from five halophilic archaeal strains, J. Ind. Microbiol. Biotechnol. 36 (2009) 105–110, https://doi.org/10.1007/s10295-008-0477-8.

[19] L. Ramnath, B. Sithole, R. Govinden, Identification of lipolytic enzymes isolated from bacteria indigenous to Eucalyptus wood species for application in the pulping industry, Biotechnol. Rep. 15 (2017) 114–124, https://doi.org/10.1016/j.btre.2017.07.004.

[20] C.L. Teo, H. Jamaluddin, N.A.M. Zain, A. Idris, Biodiesel production via lipase catalysed transesterification of microalgae lipids from Tetraselmis sp, Renew. Energy 68 (2014) 1–5, https://doi.org/10.1016/J.RENENE.2014.01.027.

[21] R.A. Gross, A. Kumar, B. Kalra, Polymer synthesis by in vitro enzyme catalysis, Chem. Rev. 101 (2001) 2097–2124, https://doi.org/10.1021/cr0002590.

[22] S. Chinaglia, L.R. Chiarelli, M. Maggi, M. Rodolfi, G. Valentini, A.M. Picco, Biochemistry of lipolytic enzymes secreted by *Penicillium solitum* and *Cladosporium cladosporioides*, Biosc. Biotech. Biochem. 78 (2014) 245–254, https://doi.org/10.1080/09168451.2014.882752.

[23] S.C.B. Gopinath, P. Anbu, T. Lakshmipriya, A. Hilda, Strategies to characterize fungal lipases for applications in medicine and dairy industry, BioMed Res. Int. 2013 (2013) 154549, https://doi.org/10.1155/2013/154549.

[24] F. Hasan, A.A. Shah, A. Hameed, Industrial applications of microbial lipases, Enzym. Microb. Technol. 39 (2006) 235–251, https://doi.org/10.1016/J.ENZMICTEC.2005.10.016.

[25] K.-E. Jaeger, T. Eggert, Lipases for biotechnology, Curr. Opin. Biotechnol. 13 (2002) 390–397, https://doi.org/10.1016/S0958-1669(02)00341-5.

[26] Shraddha, R. Shekher, S. Sehgal, M. Kamthania, A. Kumar, Laccase: microbial sources, production, purification, and potential biotechnological applications, Enzym. Res. (2011) 217861, https://doi.org/10.4061/2011/217861 (2011).

[27] E.-S. Lin, C.-C. Wang, S.-C. Sung, Cultivating conditions influence lipase production by the edible Basidiomycete Antrodia cinnamomea in submerged culture, Enzym. Microb. Technol. 39 (2006) 98–102, https://doi.org/10.1016/J.ENZMICTEC.2005.10.002.

[28] B. Sreelatha, V. Koteswara Rao, R. Ranjith Kumar, S. Girisham, S.M. Reddy, Culture conditions for the production of thermostable lipase by Thermomyces lanuginosus, Beni-Suef Univ. J. Basic Appl. Sci. 6 (2017) 87–95, https://doi.org/10.1016/J.BJBAS.2016.11.010.

[29] N. Kuratani, H. Shinji, N. Hideo, F. Hideki, Continuous production of biodiesel fuel by enzymatic method, U.S. Patent 9 (879) (2018) 291. https://patents.google.com/patent/US9879291B2/en.

[30] W. Orlando Beys Silva, S. Mitidieri, A. Schrank, M.H. Vainstein, Production and extraction of an extracellular lipase from the entomopathogenic fungus Metarhizium anisopliae, Process Biochem. 40 (2005) 321–326, https://doi.org/10.1016/J.PROCBIO.2004.01.005.

[31] S. Shah, S. Sharma, M.N. Gupta, Biodiesel Preparation by Lipase-Catalyzed Transesterification of Jatropha Oil, 2004, https://doi.org/10.1021/ef030075z.

[32] S.T. Keera, S.M. El Sabagh, A.R. Taman, Transesterification of vegetable oil to biodiesel fuel using alkaline catalyst, Fuel 90 (2011) 42–47, https://doi.org/10.1016/J.FUEL.2010.07.046.

[33] H.-Y. Yoo, J.R. Simkhada, S.S. Cho, H. Park, S.W. Kim, C.N. Seong, J.C. Yoo, A novel alkaline lipase from Ralstonia with potential application in biodiesel production, Bioresour. Technol. 102 (2011) 6104–6111, https://doi.org/10.1016/j.biortech.2011.02.046.

[34] D. Surendhiran, M. Vijay, A.R. Sirajunnisa, Biodiesel production from marine microalga Chlorella salina using whole cell yeast immobilized on sugarcane bagasse, J. Environ. Chem. Eng. 2 (2014) 1294–1300, https://doi.org/10.1016/J.JECE.2014.05.004.

[35] D.-T. Tran, C.-L. Chen, Effect of solvents and oil content on direct transesterification of wet oil-bearing microalgal biomass of Chlorella vulgaris ESP-31 for biodiesel synthesis using immobilized lipase as the biocatalyst, Bioresour. Technol. 135 (2013) 213–221, https://doi.org/10.1016/J.BIORTECH.2012.09.101.

[36] J.Z. Chen, S. Wang, B. Zhou, L. Dai, D. Liu, W. Du, A Robust Process for Lipase-Mediated Biodiesel Production from Microalgae Lipid, 2016, https://doi.org/10.1039/c6ra07144a.

[37] A. Guldhe, P. Singh, S. Kumari, I. Rawat, K. Permaul, F. Bux, Biodiesel synthesis from microalgae using immobilized Aspergillus Niger whole cell lipase biocatalyst, Renew. Energy 85 (2016) 1002–1010, https://doi.org/10.1016/J.RENENE.2015.07.059.

[38] L. Ramnath, B. Sithole, R. Govinden, Identification of lipolytic enzymes isolated from bacteria indigenous to Eucalyptus wood species for application in the pulping industry, Biotechnol. Rep. 15 (2017) 114–124, https://doi.org/10.1016/J.BTRE.2017.07.004.

[39] J. Carrazco-Palafox, B.E. Rivera-Chavira, N. Ramírez-Baca, L.I. Manzanares-Papayanopoulos, G.V. Nevárez-Moorillón, Improved method for qualitative screening of lipolytic bacterial strains, MethodsX 5 (2018) 68–74, https://doi.org/10.1016/J.MEX.2018.01.004.

[40] D.F.M. Turati, A.F. Almeida, C.C. Terrone, J.M.F. Nascimento, C.R.F. Terrasan, G. Fernandez-Lorente, B.C. Pessela, J.M. Guisan, E.C. Carmona, Thermotolerant lipase from Penicillium sp. section Gracilenta CBMAI 1583: effect of carbon sources on enzyme production, biochemical properties of crude and purified enzyme and substrate specificity, Biocatal. Agric. Biotechnol. 17 (2019) 15–24, https://doi.org/10.1016/j.bcab.2018.10.002.

[41] R. Tripathi, J. Singh, R. kumar Bharti, I.S. Thakur, Isolation, purification and characterization of lipase from microbacterium sp. and its application in biodiesel production, Energy Procedia 54 (2014) 518–529, https://doi.org/10.1016/J.EGYPRO.2014.07.293.

[42] P. Supakdamrongkul, A. Bhumiratana, C. Wiwat, Characterization of an extracellular lipase from the biocontrol fungus, Nomuraea rileyi MJ, and its toxicity toward Spodoptera litura, J. Invertebr. Pathol. 105 (2010) 228–235, https://doi.org/10.1016/J.JIP.2010.06.011.

[43] M.M. Maia, A. Heasley, M. Camargo de Morais, E.H. Melo, M. Morais, W. Ledingham, J. Lima Filho, Effect of culture conditions on lipase production by Fusarium solani in batch fermentation, Bioresour. Technol. 76 (2001) 23–27, https://doi.org/10.1016/S0960-8524(00)00079-1.

[44] F.F. de Castro, A.B.P. Pinheiro, C.B. Nassur, I.P. Barbosa-Tessmann, Mycelium-bound lipase from a locally isolated strain of Aspergillus westerdijkiae, Biocatal. Agric. Biotechnol. 10 (2017) 321–328, https://doi.org/10.1016/J.BCAB.2017.04.009.

[45] S.-S. Yi, J.-M. Noh, Y.-S. Lee, Amino acid modified chitosan beads: improved polymer supports for immobilization of lipase from Candida rugosa, J. Mol. Catal. B Enzym. 57 (2009) 123–129, https://doi.org/10.1016/J.MOLCATB.2008.08.002.

[46] D. Palacios, M.D. Busto, N. Ortega, Study of a new spectrophotometric end-point assay for lipase activity determination in aqueous media, LWT - Food Sci. Technol. (Lebensmittel-Wissenschaft -Technol.) 55 (2014) 536–542, https://doi.org/10.1016/J.LWT.2013.10.027.

[47] R.C. Judd, 2I-Peptide mapping of protein III isolated from four strains of Neisseria gonorrhoeae. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC347578/pdf/iai00149-0232.pdf, 1982. (Accessed 12 June 2019).

[48] U.K. Laemmli, Cleavage of structural proteins during the assembly of the

head of bacteriophage T4, Nature 227 (1970) 680–685, https://doi.org/10.1038/227680a0.
[49] M. Tsuji, Y. Yokota, K. Shimohara, S. Kudoh, T. Hoshino, An application of wastewater treatment in a cold environment and stable lipase production of antarctic basidiomycetous yeast Mrakia blollopis, PloS One 8 (2013), https://doi.org/10.1371/journal.pone.0059376.
[50] R. Gaur, A. Gupta, S.K. Khare, Purification and characterization of lipase from solvent tolerant Pseudomonas aeruginosa PseA, Process Biochem. 43 (2008) 1040–1046, https://doi.org/10.1016/J.PROCBIO.2008.05.007.
[51] Ryther Guillard, f/2 Medium for Growing Diatoms : 2L Recipe, 2005, p. 2005. Techniques.
[52] J.C. Taylor, W.R. Harding, C.G.M. Archibald, A Methods Manual for the Collection, Preparation and Analysis of Diatom Samples, 2007, https://doi.org/10.2307/2963468.
[53] H. Van Heurck, Van Heurck, A Treatise on the Diatomaceae, Translated by W.E. Baxter, Citations - Diatoms of North America, 1896 (1896), https://diatoms.org/citations/van_heurck_h-1896-a_treatise_on_the_diatomaceae_translated_by_we_baxter. (Accessed 18 August 2018).
[54] R. Simonsen, Patrick Ruth, C.H.W. Reimer, The diatoms of the United States. Vol. I. Mono graphs of the academy of natural sciences of philadelphia, 13, xi + 688 pp., 64 Taf., Philadelphia, Internationale Revue Der Gesamten Hydrobiologie Und Hydrographie 53 (1966) 166–167, https://doi.org/10.1002/iroh.19680530120 (1968).
[55] X.L. Li, T.K. Marella, L. Tao, L. Peng, C.F. Song, L.L. Dai, A. Tiwari, G. Li, A novel growth method for diatom algae in aquaculture waste water for natural food development and nutrient removal, Water Sci. Technol. 75 (2017) 2777–2783, https://doi.org/10.2166/wst.2017.156.
[56] M. Axelsson, F. Gentili, A single-step method for rapid extraction of total lipids from green microalgae, PloS One 9 (2014), e89643, https://doi.org/10.1371/journal.pone.0089643.
[57] R. Jambulingam, M. Shalma, V. Shankar, Biodiesel production using lipase immobilised functionalized magnetic nanocatalyst from oleaginous fungal lipid, J. Clean. Prod. 215 (2019) 245–258, https://doi.org/10.1016/J.JCLEPRO.2018.12.146.
[58] L. Zhu, Z. Wang, Q. Shu, J. Takala, E. Hiltunen, P. Feng, Z. Yuan, Nutrient removal and biodiesel production by integration of freshwater algae cultivation with piggery wastewater treatment, Water Res. 47 (2013) 4294–4302, https://doi.org/10.1016/J.WATRES.2013.05.004.
[59] B. Bharathiraja, R. Ranjith Kumar, R. PraveenKumar, M. Chakravarthy, D. Yogendran, J. Jayamuthunagai, Biodiesel production from different algal oil using immobilized pure lipase and tailor made rPichia pastoris with Cal A and Cal B genes, Bioresour. Technol. 213 (2016) 69–78, https://doi.org/10.1016/J.BIORTECH.2016.02.041.
[60] A.F. Panichikkal, P. Prakasan, U. Kizhakkepowathial Nair, M. Kulangara Valappil, Optimization of parameters for the production of biodiesel from rubber seed oil using onsite lipase by response surface methodology, 3 Biotech 8 (2018) 459, https://doi.org/10.1007/s13205-018-1477-7.
[61] K. Ramluckan, K.G. Moodley, F. Bux, An evaluation of the efficacy of using selected solvents for the extraction of lipids from algal biomass by the soxhlet extraction method, Fuel 116 (2014) 103–108, https://doi.org/10.1016/J.FUEL.2013.07.118.
[62] C.A.L. Abe, C.B. Faria, F.F. de Castro, S.R. de Souza, F.C. dos Santos, C.N. da Silva, D.J. Tessmann, I.P. Barbosa-Tessmann, Fungi isolated from maize (Zea mays L.) grains and production of associated enzyme activities, Int. J. Mol. Sci. 16 (2015) 15328–15346, https://doi.org/10.3390/ijms160715328.
[63] S. Chinaglia, L.R. Chiarelli, M. Maggi, M. Rodolfi, G. Valentini, A.M. Picco, Biochemistry of lipolytic enzymes secreted by Penicillium solitum and Cladosporium cladosporioides, Biosci. Biotechnol. Biochem. 78 (2014) 245–254, https://doi.org/10.1080/09168451.2014.882752.
[64] P. Hong, S. Koza, E.S.P. Bouvier, Size-exclusion chromatography for the analysis of protein biotherapeutics and their aggregates, J. Liq. Chromatogr. Relat. Technol. 35 (2012) 2923–2950, https://doi.org/10.1080/10826076.2012.743724.
[65] P. Supakdamrongkul, A. Bhumiratana, C. Wiwat, Characterization of an extracellular lipase from the biocontrol fungus, Nomuraea rileyi MJ, and its toxicity toward Spodoptera litura, J. Invertebr. Pathol. 105 (2010) 228–235, https://doi.org/10.1016/j.jip.2010.06.011.
[66] W. Stöcklein, H. Sztajer, U. Menge, R.D. Schmid, Purification and properties of a lipase from Penicillium expansum, Biochim. Biophys. Acta Lipids Lipid. Metabol. 1168 (1993) 181–189, https://doi.org/10.1016/0005-2760(93)90123-Q.
[67] M. Yiğitoğlu, Z. Temoçin, Immobilization of Candida rugosa lipase on glutaraldehyde-activated polyester fiber and its application for hydrolysis of some vegetable oils, J. Mol. Catal. B Enzym. 66 (2010) 130–135, https://doi.org/10.1016/J.MOLCATB.2010.04.007.
[68] C.M. Romero, M.D. Baigori, L.M. Pera, Catalytic properties of mycelium-bound lipases from Aspergillus Niger MYA 135, Appl. Microbiol. Biotechnol. 76 (2007) 861–866, https://doi.org/10.1007/s00253-007-1067-9.
[69] A. Salihu, M. Zahangir Alam, M. Ismail AbdulKarim, H.M. Salleh, Effect of process parameters on lipase production by Candida cylindracea in stirred tank bioreactor using renewable palm oil mill effluent based medium, J. Mol. Catal. B Enzym. 72 (2011) 187–192, https://doi.org/10.1016/j.molcatb.2011.06.004.
[70] F.F. de Castro, A.B.P. Pinheiro, C.B. Nassur, I.P. Barbosa-Tessmann, Mycelium-bound lipase from a locally isolated strain of Aspergillus westerdijkiae, Biocatal. Agric. Biotechnol. 10 (2017) 321–328, https://doi.org/10.1016/J.BCAB.2017.04.009.
[71] M. Essamri, V. Deyris, L. Comeau, Optimization of lipase production by Rhizopus oryzae and study on the stability of lipase activity in organic solvents, J. Biotechnol. 60 (1998) 97–103, https://doi.org/10.1016/S0168-1656(97)00193-4.
[72] M. Naushad, Z.A. ALOthman, A.B. Khan, M. Ali, Effect of ionic liquid on activity, stability, and structure of enzymes: a review, Int. J. Biol. Macromol. 51 (2012) 555–560, https://doi.org/10.1016/J.IJBIOMAC.2012.06.020.
[73] P. Supakdamrongkul, A. Bhumiratana, C. Wiwat, Characterization of an extracellular lipase from the biocontrol fungus, Nomuraea rileyi MJ, and its toxicity toward Spodoptera litura, J. Invertebr. Pathol. 105 (2010) 228–235, https://doi.org/10.1016/j.jip.2010.06.011.
[74] A. Hiol, M.D. Jonzo, N. Rugani, D. Druet, L. Sarda, L.C. Comeau, Purification and characterization of an extracellular lipase from a thermophilic Rhizopus oryzae strain isolated from palm fruit, Enzym. Microb. Technol. 26 (2000) 421–430, https://doi.org/10.1016/S0141-0229(99)00173-8.
[75] M. Yu, S. Qin, T. Tan, Purification and characterization of the extracellular lipase Lip2 from Yarrowia lipolytica, Process Biochem. 42 (2007) 384–391, https://doi.org/10.1016/J.PROCBIO.2006.09.019.
[76] K. Krammer, H. Bertolet, K. Krammer, H. Lange-Bertalot, Bacillariophyceae. 1. Teil: naviculaceae, in: H. Ettl, J. Gerloff, H. Heynig, D. Mollenhauer (Eds.), Süsswasserflora von Mitteleuropa, 1986. Band 2/1., 1986.
[77] J. Pruvost, G. Van Vooren, B. Le Gouic, A. Couzinet-Mossion, J. Legrand, Systematic investigation of biomass and lipid productivity by microalgae in photobioreactors for biodiesel application, Bioresour. Technol. 102 (2011) 150–158, https://doi.org/10.1016/J.BIORTECH.2010.06.153.
[78] C.A. Popovich, M. Pistonesi, P. Hegel, D. Constenla, G.B. Bielsa, L.A. Martín, M.C. Damiani, P.I. Leonardi, Unconventional alternative biofuels: quality assessment of biodiesel and its blends from marine diatom Navicula cincta, Algal Res. 39 (2019) 101438, https://doi.org/10.1016/J.ALGAL.2019.101438.
[79] M.J. Griffiths, R.P. van Hille, S.T.L. Harrison, Lipid productivity, settling potential and fatty acid profile of 11 microalgal species grown under nitrogen replete and limited conditions, J. Appl. Phycol. 24 (2012) 989–1001, https://doi.org/10.1007/s10811-011-9723-y.
[80] T.K. Marella, N.R. Parine, A. Tiwari, Potential of diatom consortium developed by nutrient enrichment for biodiesel production and simultaneous nutrient removal from waste water, Saudi J. Biol. Sci. 25 (2018) 704–709, https://doi.org/10.1016/J.SJBS.2017.05.011.
[81] M.M. Joseph, K.R. Renjith, G. John, S.M. Nair, N. Chandramohanakumar, Biodiesel prospective of five diatom strains using growth parameters and fatty acid profiles, Biofuels 8 (2017) 81–89, https://doi.org/10.1080/17597269.2016.1204585.
[82] G.B. Bielsa, C.A. Popovich, M.C. Rodríguez, A.M. Martínez, L.A. Martín, M.C. Matulewicz, P.I. Leonardi, Simultaneous production assessment of triacylglycerols for biodiesel and exopolysaccharides as valuable co-products in Navicula cincta, Algal Res. 15 (2016), https://doi.org/10.1016/j.algal.2016.01.013.
[83] G. Saranya, M.D. Subashchandran, P. Mesta, T.V. Ramachandra, Prioritization of prospective third-generation biofuel diatom strains, Energy, Ecol. Environ. 3 (2018) 338–354, https://doi.org/10.1007/s40974-018-0105-z.
[84] F. Mus, J.-P. Toussaint, K.E. Cooksey, M.W. Fields, R. Gerlach, B.M. Peyton, R.P. Carlson, Physiological and Molecular Analysis of Carbon Source Supplementation and pH Stress-Induced Lipid Accumulation in the Marine Diatom Phaeodactylum Tricornutum, 2013, https://doi.org/10.1007/s00253-013-4747-7.
[85] G. Knothe, Dependence of biodiesel fuel properties on the structure of fatty acid alkyl esters, Fuel Process. Technol. 86 (2005) 1059–1070, https://doi.org/10.1016/J.FUPROC.2004.11.002.
[86] T.V. Ramachandra, D.M. Mahapatra, K.B., R. Gordon, Milking diatoms for sustainable energy: biochemical engineering versus gasoline-secreting diatom solar panels, Ind. Eng. Chem. Res. 48 (2009) 8769–8788, https://doi.org/10.1021/ie900044j.
[87] D.M. Mahapatra, H.N. Chanakya, T.V. Ramachandra, Bioremediation and lipid synthesis through mixotrophic algal consortia in municipal wastewater, Bioresour. Technol. 168 (2014) 142–150, https://doi.org/10.1016/j.biortech.2014.03.130.
[88] C.L. Teo, H. Jamaluddin, N.A.M. Zain, A. Idris, Biodiesel production via lipase catalysed transesterification of microalgae lipids from Tetraselmis sp, Renew. Energy 68 (2014) 1–5, https://doi.org/10.1016/J.RENENE.2014.01.027.
[89] X.-Q. Xu, J. Beardall, Effect of salinity on fatty acid composition of a green microalga from an antarctic hypersaline lake, Phytochemistry 45 (1997) 655–658, https://doi.org/10.1016/S0031-9422(96)00868-0.
[90] G.G. Satpati, P. Chandra Gorain, I. Paul, R. Pal, An integrated salinity-driven workflow for rapid lipid enhancement in green microalgae for biodiesel application, RSC Adv. 6 (2016) 112340–112355, https://doi.org/10.1039/C6RA23933A.
[91] S. AS, Biodiesel and polyunsaturated fatty acid (PUFA) potential of MicroalgaeBiomass-A short review, Res. Dev. Mater. Sci. 10 (2019), https://doi.org/10.31031/rdms.2019.10.000744.
[92] I.A. Nascimento, S.S.I. Marques, I.T.D. Cabanelas, S.A. Pereira, J.I. Druzian, C.O. de Souza, D.V. Vich, G.C. de Carvalho, M.A. Nascimento, Screening microalgae strains for biodiesel production: lipid productivity and estimation of fuel quality based on fatty acids profiles as selective criteria, BioEnergy Res. 6 (2013) 1–13, https://doi.org/10.1007/s12155-012-9222-2.

[93] P.M. Schenk, S.R. Thomas-Hall, E. Stephens, U.C. Marx, J.H. Mussgnug, C. Posten, O. Kruse, B. Hankamer, Second generation biofuels: high-efficiency microalgae for biodiesel production, BioEnergy Res. 1 (2008) 20–43, https://doi.org/10.1007/s12155-008-9008-8.
[94] T. Veeranan, R. Kasirajaan, B. Gurunathan, R. Sahadevan, A novel approach for extraction of algal oil from marine macroalgae Ulva fasciata, Renew. Energy 127 (2018) 64–73, https://doi.org/10.1016/J.RENENE.2017.12.071.
[95] M.A. Njoki, G. Mercy, G. Nyagah, A. Gachanja, Fourier Transform Infrared Spectrophotometric Analysis of Functional Groups Found in Ricinus Communis L. And Cucurbita Maxima Lam. Roots, Stems and Leaves as Heavy Metal Adsorbents, 2016. www.ijset.net. (Accessed 24 July 2019).
[96] S. Bargole, S. George, V. Kumar Saharan, Improved rate of transesterification reaction in biodiesel synthesis using hydrodynamic cavitating devices of high throat perimeter to flow area ratios, Chem. Eng. Proc. - Process Intensification 139 (2019) 1–13, https://doi.org/10.1016/J.CEP.2019.03.012.
[97] A.Y. Oyerinde, E.I. Bello, E. Lanchares Sancho, Article no.BJAST.22178 reviewers: (1) J. Gimbun, original research article oyerinde and bello, BJAST (2016) 1–14, https://doi.org/10.9734/BJAST/2016/22178.
[98] J.T. Kloprogge, R.D. Schuiling, Z. Ding, L. Hickey, D. Wharton, R.L. Frost, Vibrational spectroscopic study of syngenite formed during the treatment of liquid manure with sulphuric acid, Vib. Spectrosc. 28 (2002) 209–221, https://doi.org/10.1016/S0924-2031(01)00139-4.
[99] A.F. Ferreira, A.P.S. Dias, C.M. Silva, M. Costa, Bio-oil and bio-char characterization from microalgal biomass, V Confer{ê}ncia Nacional de Mec{â}nica Dos Fluidos, Termodin{â}mica e Energia (Fifth National Conference on Fluid Mechanics, Thermodynamics and Energy), 2014, pp. 11–12. http://paginas.fe.up.pt/~mefte2014/wp-content/uploads/2014/preprint/mefte2014_submission_45.pdf.
[100] A. Adenike Evelyn, O. Abiodun Oluwafemi, Spectroscopic analysis of oil extracted from seeds of hildegardia barteri (Mast.) kosterm, 2018, https://doi.org/10.4172/2329-6836.1000325.
[101] R. Tripathi, J. Singh, R. kumar Bharti, I.S. Thakur, Isolation, purification and characterization of lipase from microbacterium sp. and its application in biodiesel production, Energy Procedia 54 (2014) 518–529, https://doi.org/10.1016/J.EGYPRO.2014.07.293.
[102] A. Guldhe, B. Singh, I. Rawat, K. Permaul, F. Bux, Biocatalytic conversion of lipids from microalgae Scenedesmus obliquus to biodiesel using Pseudomonas fluorescens lipase, Fuel 147 (2015) 117–124, https://doi.org/10.1016/j.fuel.2015.01.049.
[103] C.G. Lopresto, S. Naccarato, L. Albo, M.G. De Paola, S. Chakraborty, S. Curcio, V. Calabrò, Enzymatic transesterification of waste vegetable oil to produce biodiesel, Ecotoxicol. Environ. Saf. 121 (2015) 229–235, https://doi.org/10.1016/J.ECOENV.2015.03.028.
[104] J.Z. Chen, S. Wang, B. Zhou, L. Dai, D. Liu, W. Du, A robust process for lipase-mediated biodiesel production from microalgae lipid, RSC Adv. 6 (2016) 48515–48522, https://doi.org/10.1039/C6RA07144A.
[105] B. Scholz, G. Liebezeit, Biochemical characterisation and fatty acid profiles of 25 benthic marine diatoms isolated from the Solthörn tidal flat (southern North Sea), J. Appl. Phycol. 25 (2013) 453–465, https://doi.org/10.1007/s10811-012-9879-0.
[106] C. Delgado, I. Pardo, L. García, Diatom communities as indicators of ecological status in Mediterranean temporary streams (Balearic Islands, Spain), Ecol. Indicat. 15 (2012) 131–139, https://doi.org/10.1016/J.ECOLIND.2011.09.037.
[107] F. in the T.L.C. and B.C. of D.S. for P.B.P Zhao, J. Liang, Y. Gao, Q. Luo, Y. Yu, C. Chen, L. Sun, Variations in the total lipid content and biological characteristics of diatom species for potential biodiesel production, Fund. Renew. Energy Appl. 6 (2016) 22–26, https://doi.org/10.4172/20904541.1000201.
[108] F.J. Fields, J.P. Kociolek, An evolutionary perspective on selecting high-lipid-content diatoms (Bacillariophyta), J. Appl. Phycol. 27 (2015) 2209–2220, https://doi.org/10.1007/s10811-014-0505-1.
[109] S. Hausmann, D.F. Charles, J. Gerritsen, T.J. Belton, A diatom-based biological condition gradient (BCG) approach for assessing impairment and developing nutrient criteria for streams, Sci. Total Environ. 562 (2016) 914–927, https://doi.org/10.1016/j.scitotenv.2016.03.173.
[110] M.R. De La Peña, Cell growth and nutritive value of the tropical benthic diatom, Amphora sp., at varying levels of nutrients and light intensity, and different culture locations, Springer Verlag, J. Appl. Phycol. (2007) 647–655, https://doi.org/10.1007/s10811-007-9189-0.
[111] G. d'Ippolito, A. Sardo, D. Paris, F.M. Vella, M.G. Adelfi, P. Botte, C. Gallo, A. Fontana, Potential of lipid metabolism in marine diatoms for biofuel production, Biotechnol. Biofuels 8 (2015) 28, https://doi.org/10.1186/s13068-015-0212-4.
[112] J.C. Taylor, J. Prygiel, A. Vosloo, P.A. de la Rey, L. van Rensburg, Can diatom-based pollution indices be used for biomonitoring in South Africa? A case study of the Crocodile West and Marico water management area, Hydrobiologia 592 (2007) 455–464, https://doi.org/10.1007/s10750-007-0788-1.
[113] L. Chen, T. Liu, W. Zhang, X. Chen, J. Wang, Biodiesel production from algae oil high in free fatty acids by two-step catalytic conversion, Bioresour. Technol. 111 (2012) 208–214, https://doi.org/10.1016/J.BIORTECH.2012.02.033.
[114] J. Sheehan, T. Dunahay, J. Benemann, P. Roessler, Look Back at the U.S. Department of Energy's Aquatic Species Program: Biodiesel from Algae, 1998, https://doi.org/10.2172/15003040. Close-Out Report.
[115] X. Tan, Q. Zhang, M.A. Burford, F. Sheldon, S.E. Bunn, Benthic diatom based indices for water quality assessment in two subtropical streams, Front. Microbiol. 8 (2017) 601. https://www.frontiersin.org/article/10.3389/fmicb.2017.00601.
[116] X. Tan, X. Xia, Q. Zhao, Q. Zhang, Temporal variations of benthic diatom community and its main influencing factors in a subtropical river, China, Environ. Sci. Pollut. Control Ser. 21 (2014) 434–444, https://doi.org/10.1007/s11356-013-1898-0.
[117] M. Branco-Vieira, S. San Martin, C. Agurto, M. Santos, M. Freitas, T. Mata, A. Martins, N. Caetano, M. Branco-Vieira, S. San Martin, C. Agurto, M.A. dos Santos, M.A.V. Freitas, T.M. Mata, A.A. Martins, N.S. Caetano, Potential of phaeodactylum tricornutum for biodiesel production under natural conditions in Chile, Energies 11 (2017) 54, https://doi.org/10.3390/en11010054.
[118] A. Hiol, M.D. Jonzo, D. Druet, L. Comeau, Production, purification and characterization of an extracellular lipase from Mucor hiemalis f. hiemalis, Enzym. Microb. Technol. 25 (1999) 80–87, https://doi.org/10.1016/S0141-0229(99)00009-5.
[119] M.D. Jonzo, A. Hiol, D. Druet, L.C. Comeau, Application of immobilized lipase fromCandida rugosa to synthesis of cholesterol oleate, J. Chem. Technol. Biotechnol. 69 (1997) 463–469, https://doi.org/10.1002/(SICI)1097-4660(199708)69:4<463::AID-JCTB738>3.0.CO;2-2.
[120] H. Noureddini, X. Gao, R.S. Philkana, Immobilized Pseudomonas cepacia lipase for biodiesel fuel production from soybean oil, Bioresour. Technol. 96 (2005) 769–777, https://doi.org/10.1016/j.biortech.2004.05.029.
[121] S. Shah, S. Sharma, M.N. Gupta, Biodiesel Preparation by Lipase-Catalyzed Transesterification of Jatropha Oil, 2004, https://doi.org/10.1021/ef030075z.
[122] K. Nie, F. Xie, F. Wang, T. Tan, Lipase catalyzed methanolysis to produce biodiesel: optimization of the biodiesel production, J. Mol. Catal. B Enzym. 43 (2006) 142–147, https://doi.org/10.1016/J.MOLCATB.2006.07.016.
[123] D.T. Tran, C.L. Chen, J.S. Chang, Effect of solvents and oil content on direct transesterification of wet oil-bearing microalgal biomass of Chlorella vulgaris ESP-31 for biodiesel synthesis using immobilized lipase as the biocatalyst, Bioresour. Technol. (2013), https://doi.org/10.1016/j.biortech.2012.09.101.
[124] M. Xiao, R. Intan, J.P. Obbard, Biodiesel production from microalgae oil-lipid feedstock via immobilized whole-cell biocatalysis, in: Proceedings Venice, Third International Symposium on Energy from Biomass and Waste, Venice, Italy, 2010, pp. 8–11.