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Green microalgal strain *Chlorella vulgaris* isolated from industrial wastewater with remediation capacity

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ABSTRACT

The present work primarily focused on isolation of *Chlorella vulgaris* from industrial wastewater, and use as an effective feedstock for producing renewable biodiesel. Post isolation, the lipid was extracted from *Chlorella vulgaris* using Soxhlet's extraction method; and the extracted lipid was converted into algal oil biodiesel, which was then characterized using GC–MS spectral analysis. From the optimized reaction parameters: reaction temperature (45 °C), methanol/ C. vulgaris bio-oil ratio (4:1), catalyst concentration (300 mg) of synthesized lipase immobilized magnetic nanoparticles, and reaction time (6 h) under continuous stirring, the highest yield of C. vulgaris biodiesel was recorded as 87.6%. Besides, the lipase immobilized nano-bio catalyst used in the production process, was found to be highly efficient for about 5 to 6 cycles without any significant loss in the conversion efficiency. Finally, the evaluated fuel properties of the produced *C. vulgaris* biodiesel were in good agreement with ASTM D6751 standards.

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1. Introduction

In this twenty-first century, major research interest have been projected on the advancement of the refineries to implement cheaper fossil feedstocks such as coal and crude oil refined products (petroleum, diesel and natural gas) for commercial applications. Even though, these fossil resources are used as fuel; they are deemed unworthy to be tagged as renewable, sustainable, environmentally friendly and cheap sourced fuel. Furthermore, burning of these fossil fuels releases a large amount of CO_2 into the atmosphere; which leads to global warming and results in climate change, environmental pollution, global warming (Forján et al., 2011). In fact, this rise in the concentration of greenhouse gases was primarily contributed by industrialization and other human activities. Unfortunately, these global changes have posed threat to the environment and have raised challenges for the human existence. In order to rectify these phenomenal changes, renewable energy resources produced from sunlight, geothermal sources, biomass, tidal-oceanic waves and wind are being used, instead of the conventional fossil fuels. Among these suggested resources, biofuel from biomass is always regarded as highly feasible on account of its energy density and simplicity (Mostafa, 2010). To begin with, bio-oils and fats extracted from these biomasses have good properties and sound promising for making good quality renewable and sustainable biodiesel (Munir et al., 2020; Srinivasan et al., 2020). Eventually, these biodiesel are easily miscible with petroleum diesel for any blending proportion; and exhibits reduced emission of carbon and sulfur compounds than compared to fossil fuels (Srinivasan et al., 2020; Rajivgandhi et al., 2021). Nowadays, algal biomass, especially both microalgae and macroalgae have been seen as the major spotlight for biodiesel production, as algal cells stores a higher concentration of lipids; that can be converted to biodiesel by trans-esterification process (Shalini et al., 2021). In fact, biodiesel from these algae are found to be highly sustainable and feasible. Generally, microalgae are most predominantly found in both water bodies and terrestrial areas; and use photosynthesis for converting CO_2 and light into biomass with high energy content made up of valuable products such as carbohydrates, pigments and lipid. Moreover, supplying adequate nutrients and CO_2 to the algae, in addition to maintaining optimum pH, temperature and light source, ensures better growth; whereas, nutrient starvation results in increased production of lipids. Besides, genetic modification increases the lipid accumulation in algae (Elumalai et al., 2011; Mohanraj et al., 2021). In addition, these microalgae can be grown in wastewater, and utilizes the nutrients available in the wastewater for their metabolic functions; thereby making them suitable for wastewater treatment applications. Also, Heredia et al. (2021) suggested that, microalgae have the advantage of growing in marginal and non-arable lands with limited water supply, than compared to plants. Thus, the utilization of microalgae for energy applications can be seen as effective solution for simultaneously reducing the carbon emission and rectifying the energy crisis. Presently, this research study focus on isolating a green microalgal strain from wastewater and was later used for biodiesel production using a nano biocatalyst.

2. Materials and methods

2.1. Procurement of chemicals

All the necessary chemicals used in the study were purchased from Hi-media Laboratory, Mumbai, India. The purchased chemicals include (in alphabetical order) ammonium acetate, APTES (3-Aminopropyl)-triethoxysilane, calcium chloride, cetyl trimethyl ammonium bromide, chloroform, deionized water, diethyl ether, EDC (1-Ethyl-3-(Dimethylamino propyl)-carbodiimide), ethanol acetone, ethanol, ethylene diamine tetraacetic acid, ferric chloride hexahydrate, ferrous sulfate heptahydrate, gum Arabic, hexane, hydrochloric acid, isoamyl alcohol, isopropanol, lipase enzyme, methanol, N-Hydroxysuccinimide, Nile red stain, olive oil, potassium hydroxide, potassium iodide, sodium chloride, sodium dodecyl sulfate, sodium hydroxide, sodium hydroxide, sodium taurocholate, sulfuric acid were used for the study.

2.2. Isolation of microalgal strain from industrial wastewater

The water samples containing visible micro-algal populations were collected from the Saptarishi agro-industrial wastewater plant which is located in Kancheepuram district, Tamil Nadu, India. The samples were collected at the upper and lower layer of water at two different locations. Post collection, the samples were used for isolating the high lipid-producing micro algal strains; wherein the microalgae populations were screened from the samples by the serial dilution technique using Bristol's modified medium agar plates. After plating, the Petri plates were kept in a greenhouse at a controlled temperature of 28 °C for about 14 days. After incubation, every colonial morphology of the plate was observed under the compound microscope.

2.3. Screening of oleaginous microalgal strain

The screening of high lipid-containing microalgal strain was carried out based on the Nile red staining method. For this purpose, the microalgal strain was centrifuged at 1500 rpm for 10 min, and then the pellets (microalgal cells) were washed in 0.5 mL saline water several times, in order to remove unsuspended particles. Following this, the microalgal cells were treated with 0.5 mL of Nile red solution and were incubated for about 10 min at 37 °C for about 14 days (Zhu et al., 2017). Post incubation, the stained microalgal cells were washed in distilled water successively to eliminate the unstained dye particles; and the intracellular lipid contents were observed by fluorescence microscope at a wavelength of 470 nm (Elumalai et al., 2011; Matsunaga et al., 2009).

2.4. Molecular characterization of Chlorella Vulgaris

Species-level identification of the selected microalgal strain were confirmed by molecular characterization using the ITS region; and for the purpose of genotyping, DNA was extracted from the algal sample (Yang et al., 2013). For this purpose, the microalgal pellet was treated with 500 μL of standard Tris-EDTA buffer (10 mM Tris–HCl, pH 8.0 and 1 mM EDTA, pH 8.0) 100 μL of 1% SDS, 100 μL of lysozyme and 50 μl of proteinase K. Following this, the whole mixture was incubated at 70 °C for an hour; and after incubation, 0.5 mL of chloroform: phenol (1:5) was added to this mixture and was mixed well thoroughly, followed by the supernatant being collected after centrifugation. The total DNA in the supernatant was precipitated directly by the addition of absolute ethanol, and was washed two times in 70% ethanol; and was then centrifuged. The pellet was re-excluded in Tris-EDTA buffer and stored at −2 °C. The genomic DNA was amplified using primers targeting the internal transcribed spacer (ITS) region. The forward and reverse primers are (ITS1:5′-TCCGTAGGTGAACCTGCGG-3′ and ITS4: 5′-TCCTCCGCTTATTGATATGC-3′) (Siahmard et al., 2017). The amplified DNA using PCR was sequenced using 18 s rRNA molecular sequencing method. Using ITS1/ITS4 primers, PCR reaction was conducted under the following conditions: initial denaturation was carried out at 94 °C for about 2 min, followed by 35 amplification cycles at 94 °C for 45 s and then final extension at 72 °C for about 10 min. Montage PCR Cleanup Kit (Millipore) was used to purify the PCR products, by removing the dNTPs and the primes which were not involved in the reaction. Post purification, ITS1/ITS4 primers were used for the sequencing of PCR products. Using an ABI PRISM® DNA polymerase (FS Enzyme) (Applied Biosystems), the sequencing reactions were performed.

2.5. Growth analysis

Growth curve of the microalgae was calculated every day. The standard curve was done to check the cell concentration by using the absorbance reading at 680 nm. The dry biomass was calculated by centrifuging the culture, followed by discarding the supernatant and drying at 60 °C in hot air oven for about 24 h.

2.6. Mixotrophic cultivation of Chlorella Vulgaris

The isolated microalgal strain *C. Vulgaris* was grown under a mixotrophic cultivation condition to enhance the overall biomass and lipid productivity. To begin with, the culture medium contained different carbon sources which included sugar molasses, fructose, lactose, sucrose, maltose and glucose; and were added at different concentrations (2 g, 4 g, 6 g, 8 g, 10 g, and 15 g) in Bold's basal medium. Following this, the culture medium was sterilized and was then later inoculated with 20% (v/v) of growing inoculums in 500 ml Erlenmeyer flasks. The growth vessels were bubbled using an air pump and were then incubated at the optimal temperature (28 °C) for 18 days under continuous illumination of light intensity of 2000 lux. To ensure increased accuracy in the results, all the experiments were carried out in triplicates. The microalgae cell proliferation was governed by observing the absorbance values at 680 nm using the UV–Visible spectroscopic technique. The yield of the biomass and lipid produced under the mixotrophic condition was calculated using the empirical formula (Kamyab et al., 2013; Hamedi et al., 2016).

2.7. Extraction of lipid from Chlorella vulgaris

The dry microalgal biomass was measured gravimetrically. Next on, 500 g of dried algal biomass was treated with chloroform: methanol (2:1), and was then sonicated for 15 min at 30 kHz. After incubation, the biomass was sealed with filter paper and followed by add in soxhlet apparatus. Consecutively, the sample was run with 12 h. using hexane as an organic solvent. The resulting liquid from the round bottom flask was separated from the Soxhlet's apparatus and the excess solvent was separated using the distillation process. The percentage of the lipid content was identified based on the bellowed equation,

$$Lipid\ Content\ (\%) = \frac{Weight\ of\ Lipid}{Weight\ of\ dry\ biomass} x100 \quad (1)$$

2.8. Physiochemical characterization

Initially, the sample was characterized by X-ray diffraction (XRD) for characterizing the crystal structure of synthesized nanoparticles, Scanning electron microscopy (SEM) for study the morphology of synthesized nanoparticles, and Gas chromatography coupled with mass spectrometry (GC–MS) for characterizing the fatty acids in bio-oil and resultant biodiesel. The wavelength (Cu Ka) was calculated by using X-ray diffraction meter with Ni filtered radiation. SEM image of magnetic nanoparticle and the lipase coated magnetic nanoparticle was recorded using Carl Zeiss SUPRA55 Field Emission Scanning Electron Microscope at a voltage of 5 kV (Jambulingam et al., 2019). Similarly, biodiesel produced from lipids of *Chlorella vulgaris* was analyzed in Agilent 6890 gas chromatograph-straight deactivated 2 mm direct injector liners and a 15 m All tech EC-5 columns (250 μ I.D., 0.25 μ film thickness). Here, the helium carrier gas was set to a 2 mL/min flow rate. Technical specifications of mass spectrometer are as follows: JEOL GC mate II benchtop double-focusing magnetic sector mass spectrometer operating in electron ionization (EI) mode with TSS-20001 software (Srinivasan et al., 2020). Lastly, the different fatty acid compounds were identified by comparing the obtained spectral peaks with the recorded spectra in the data bank mass spectra of NIST library V 11 provided by the GC–MS.

2.9. Preparation of immobilized and functionalized magnetic nanoparticle

$FeSO_4.7H_2O$ and $Fecl_3.6H_2O$ were taken together 100 mL of Deionized H_2O by co-precipitation method (Xie and Ma, 2010). Following this, the mixture was heated at 90 °C, and the reaction environment was maintained at pH 10 by adding 1M NaOH drop wise; and then, the sample was kept under vigorous stirring. In the end, produced black precipitate was washed 4–5 times with deionized water and dried overnight at 60 °C and stored.

2.9.1. Magnetic nanoparticles using lipase immobilization

In lipid immobilization, initially 0.25 g Fe_3O_4 was taken in the formed solution of 4.8 mL ethanol APTES (0.15 Ml) and sample was sonicated 1 h for 35 kHz. Continuously, the sample was mechanical stirring for 10 h by maintaining the temperature as 40 °C. Post stirring, the end product was washed with suitable solvent (in this case, ethanol), and was separated by the means of magnetic decantation. Moving on, the synthesized Fe_3O_4-APTES was dried at lower temperature (50-55 °C) inside a hot air oven. Simultaneously, about 2.5 ml of lipase solution and 0.5 ml of 2.5 mg/ml EDC solution was mixed into a mixture and was incubated at ambient temperature for 3 h under the influence of mechanical shaking maintained at 200rpm. Next up, 3 mg of NHS was added to this reaction mixture and was incubated again for the same reaction conditions. Following this, the prepared reaction solution was refluxed with 0.25 g of synthesized Fe_3O_4-APTES nanoparticles and was again incubated for 3 h. Post immobilization, the immobilized lipase was separated by magnetization, followed by water (deionized) washing until the supernatant solution was free from free lipase. Lastly, the treated immobilized catalyst was dried at room temperature; and then was used as a nano-biocatalyst for the conversion of algal lipid into biodiesel (Raita et al., 2015).

2.9.2. Lipase activity assay

Olive oil hydrolysis shows the activity of immobilized nano catalyst using olive oil as the substrate. Briefly, 10 mL of olive oil and 2 ml of phosphate buffer, 100 $mmol/L^{-1}$ at pH 7.0 and 8 ml of water. For this purpose, the sample was sonicated 15 min using 20 ml of olive oil and 165 ml of 5% gum Arabic. Continuing on, the emulsion was prepared at different concentrations such as 100, mg, 200 mg, 300 mg, 400 mg and 500 mg of immobilized enzyme, which was then added to the reaction mixture and incubated at 35 °C for 30 min. To determine the enzyme activity, the liberated fatty acids were titrated against 0.1N NaOH using phenolphthalein as an indicator (Kandasamy et al., 2010).

2.10. Biodiesel production using enzymatic transesterification reaction

The biodiesel was produced from the extracted oil by means of enzymatic transesterification using the optimized factors. For this purpose, 300 mg of immobilized lipase enzyme was added to the mixture of 5 ml hexane and the crude oil extracted from the microalgae *Chlorella Vulgaris*. To this, 1 ml of water, methanol and oil in the ratio of 4:1 were added. The reaction mixture was added into a round bottom flask, and was placed on a hot plate magnetic stirrer. The reaction setup was maintained at a reaction temperature of 45 °C for 6 h, and was stirred mechanically at 400rpm to get the desired biodiesel yields. The reusability of the synthesized enzymatic catalyst was assessed by re-iterating the transesterification reaction under the same reaction conditions using the catalyst repeatedly and calculating the percentage yield of biodiesel produced in each cycle. To be noted, the used lipase immobilized magnetic nanoparticles based catalyst was refined and treated after each enzymatic transesterification process by removing it with the magnet and washing it repeatedly (maximum, twice) with hexane and ultrapure water to remove any foreign substance. The fatty acid methyl ester (FAME) composition of the C. vulgaris biodiesel was characterized using the GC–MS and FT-IR analytical techniques, to quantify and qualitate the resultant biodiesel. Similarly, the fuel properties of the resultant *C. vulgaris* biodiesel were evaluated using the standard ASTM D6751 standard protocol; and the evaluated fuel properties includes physical properties (density, viscosity, specific gravity), thermal properties (flash point, pour point, cloud point), chemical properties (acid value, iodine value, saponification value, ash content, and water content); and the results were compared with fuel properties of neat diesel.

3. Result and discussion

3.1. Isolation and identification of Chlorella vulgaris from wastewater

Microalgal strains were isolated from industrial wastewater were found to be capable of remediating the pollutant present in the wastewater as well effective enough to be used for biofuel production. After staining with Nile Red dye, the isolates confirmed the presence of high lipid content in the isolated microalgal strain. The high lipid content containing microalgae was identified from the selected ten colonies after examining them with a fluorescent microscope; where the Nile red-stained microalgae appeared as a colored cell with yellow dots. Here, the appearance of yellow dots in the cell indicated the neutral lipid (or) triglycerides; whereas, the appearance of red color dots in the cells indicated the polar lipid and chlorophyll (Fig. 1a). Following this, the strain exhibiting the high lipid content was chosen for the study and was identified as *Chlorella Vulgaris* using 18S rDNA fragment; and was submitted to NCBI Genbank and the accession number was allotted as KY244021. Similarly, Phylogenetic analyses were conducted in MEGA 7 and 98% of sequence similarity was more similar to *Chlorella Vulgaris*. The selected algal strain did belong to *Chlorella vulgaris* and was confirmed from BLAST analysis in the NCBI and phylogeny tree (Fig. 1c).

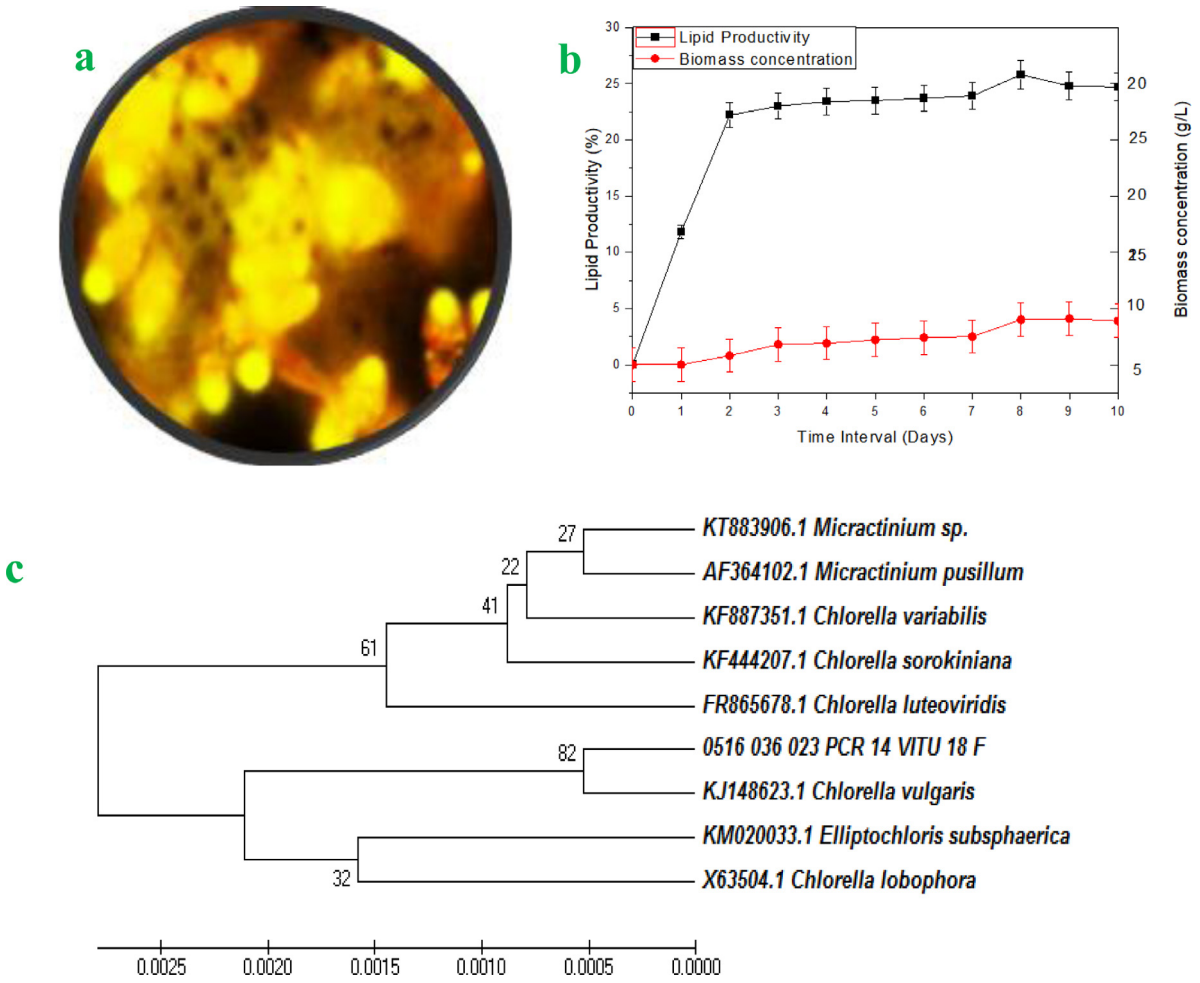


Fig. 1. Nile red staining image for the selected microalgal strains of *Chlorella vulgaris* (a) Growth curve microalgal strain of *Chlorella vulgaris* (b) and Phylogenic Tree of *Chlorella vulgaris* (c).

3.2. Growth analysis

Growth curve of the microalgae was calculated every day. Sample was withdrawn at every 24 h and calculated for dry biomass, wet biomass and lipid production. Maximum biomass and lipid production was found at 8th day with 4.8 g/L of biomass. Lipid production and biomass was found to be correlated with each other and follows the similar pattern. Biomass was found to be increased gradually and reached its peak at 8th day (Fig. 1b).

3.3. Lipid and biomass productivity

The biomass and lipid production of the selected microalgal strain *Chlorella vulgaris* was studied under the mixotrophic condition; and showed high biomass production and lipid production. Different concentration of various carbon sources were performed with Basal medium. The experimental data indicated that the selected microalgal species had shown high lipid and biomass production by utilizing the different carbon sources under mixotrophic conditions. In the mixotrophic cultivation method, sugar molasses were found to be the best carbon substrate produced maximum lipid and biomass concentration expressed in terms of a milligram per liter. *Chlorella vulgaris* showed a significant increase in the total lipid content (77%) and biomass concentration (756 mg L^{-1}), when it was grown in 10 g L^{-1} of sugar molasses (Figs. 2 and 3). From the algal biomass, lipid was extracted using soxhlet extraction process. For biodiesel production, nanobiocatalyst of transesterification was done. The lipase immobilized magnetic nano particle was used as a nano biocatalysts in the conversion of microalgal lipid into biodiesel.

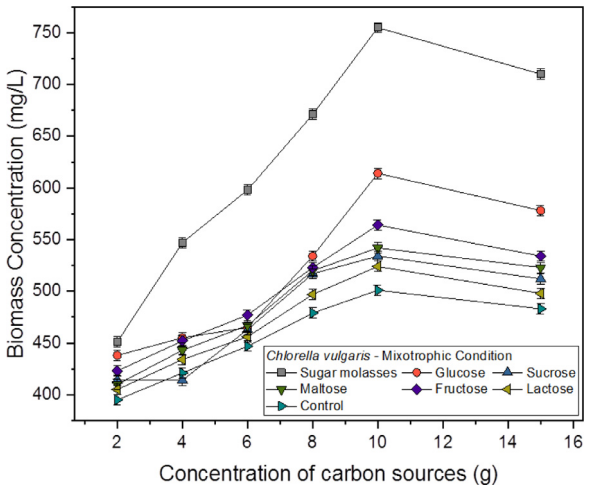


Fig. 2. Effect of carbon sources on biomass concentration of *Chlorella vulgaris* in mixotrophic condition.

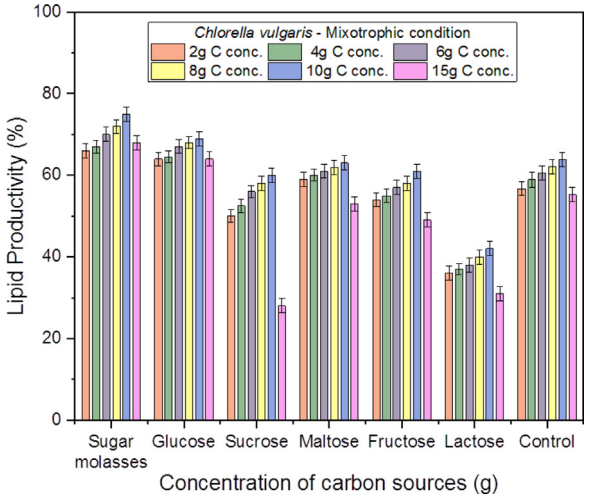


Fig. 3. Effect of carbon sources on lipid concentration of *Chlorella vulgaris* in mixotrophic condition.

3.4. Characterization of magnetic nanoparticle

The morphology of the synthesized magnetic nanoparticle was validated by SEM with electron beam energy of 5 kV. In general, normal, pure surface of the magnetic nanoparticles and mature structural surface with clear morphology of synthesized magnetic nanoparticles were observed by SEM. The Fig. 4a of lipase immobilized on the synthesized magnetic nanoparticle reported rough surfaces (Fig. 4c). From XRD results, the peaks at 2 Ø= 30.2°, 35.6°, 43.3°, 57.3°, and 62.9° were assigned to (220), (311), (400), (422), (511) and (440) reflections respectively are the characteristic peaks of the Fe_3O_4 with a cubic structure (Fig. 4b).

3.5. Characterization of immobilized lipase

The immobilized lipase with Functionalized Fe_3O_4 magnetic nanoparticle was synthesized successfully using APTES through EDC and NHS. Immobilized enzyme integrated with Fe_3O_4 magnetic nanoparticles made the surface rough due to protein present in the enzyme, which forms a layer in the surface of the nanoparticles (Fig. 4c).

3.6. Reusability and storage stability of immobilized lipase

After five time repetition, the magnetically decanted catalyst of the sample was shown absence of changes. Surprisingly, more than 5 times, the potential reduction of enzyme was clearly found. In first five times, the enzyme range was found

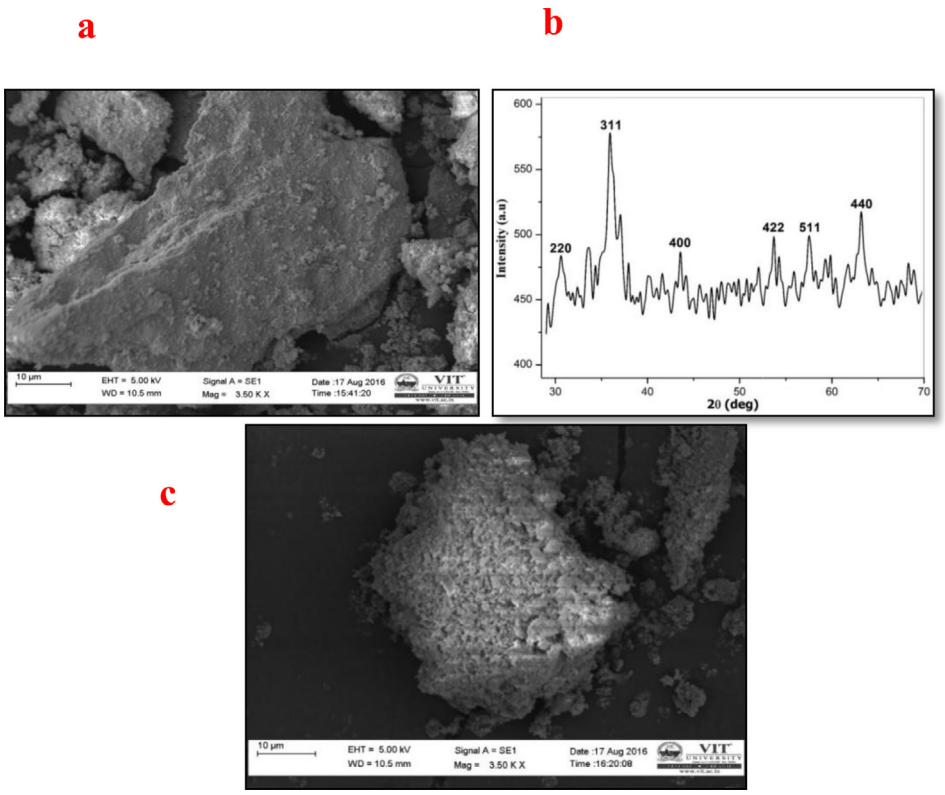


Fig. 4. SEM Image for the synthesized magnetic nanoparticles (a) XRD pattern for the synthesized magnetic nanoparticles (b) and SEM image of lipase immobilized magnetic nanoparticles (c).

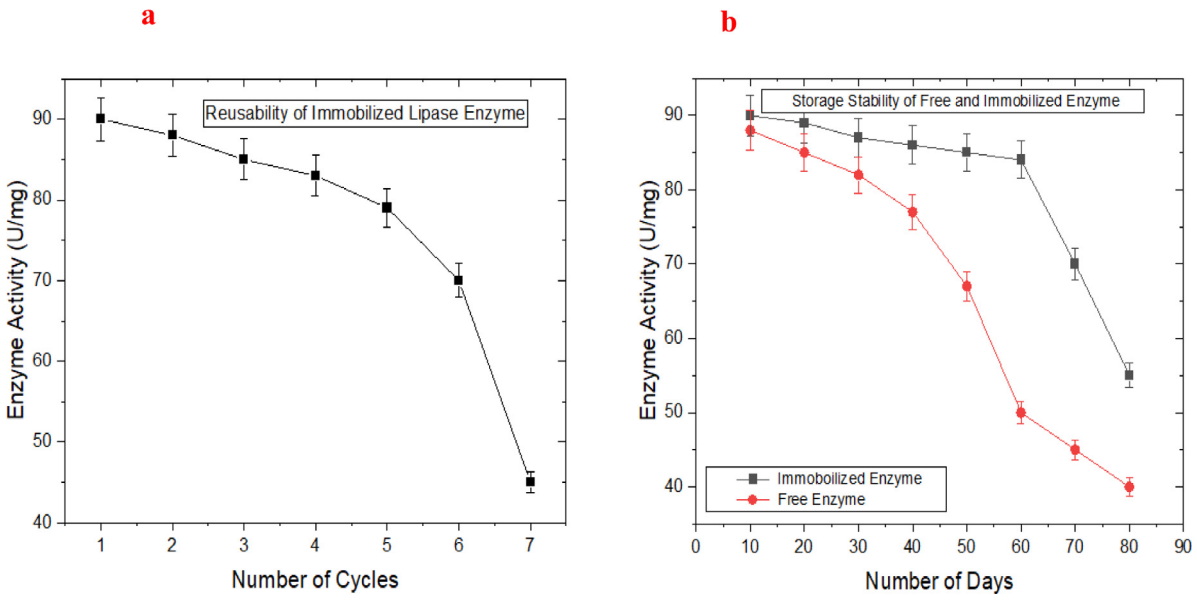


Fig. 5. Reusability and storage stability of immobilized lipase enzyme.

between the range of 88–79 U/mg (Fig. 5a) and it concluded that the immobilized enzyme was exhibited highest storage than free enzymes. Importantly, the activity was remain continues after 60 days also (Fig. 5b).

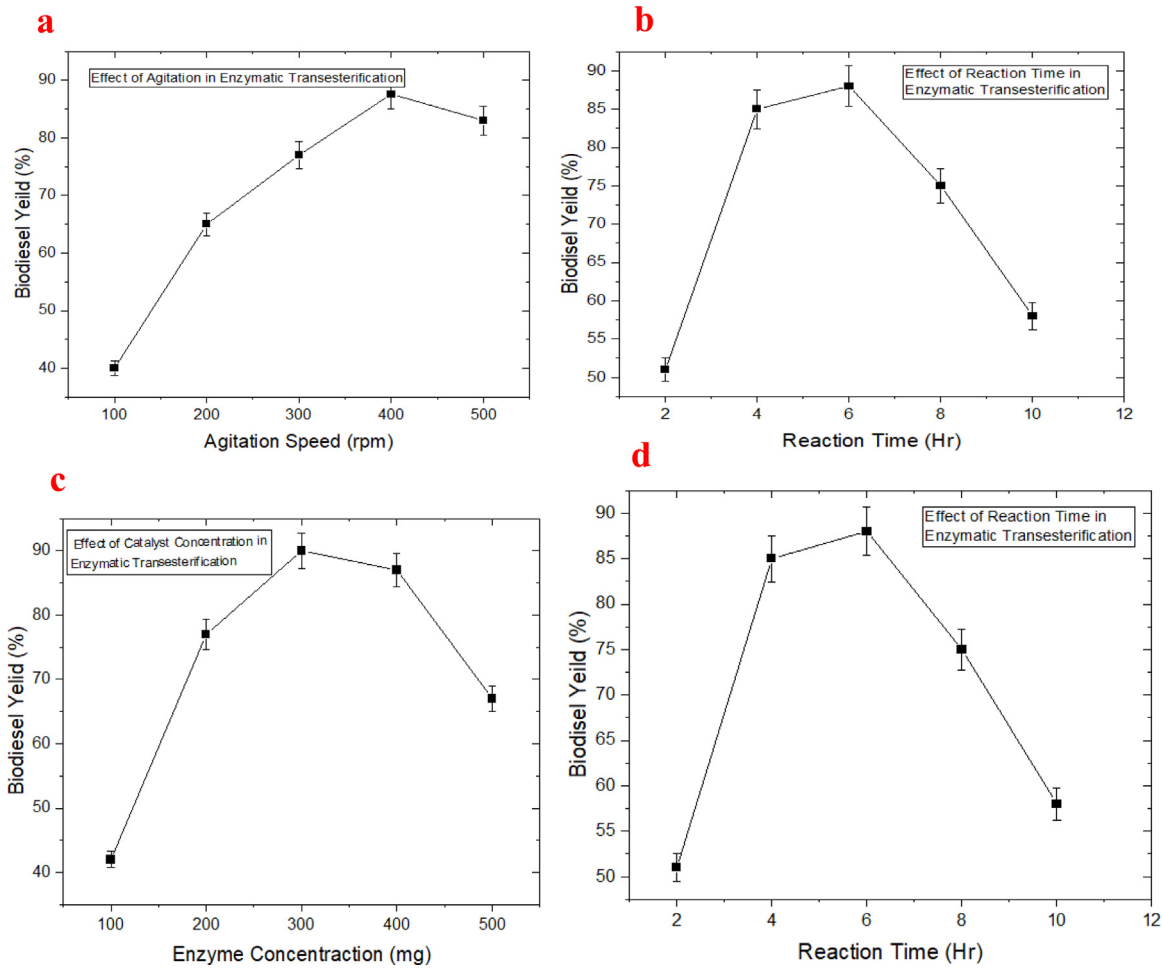


Fig. 6. Effect of different parameters in enzymatic transesterification (a, d).

3.7. Effect of oil to methanol ratio in enzymatic transesterification

The role of methanol in biodiesel production was studied by optimizing the methanol. Initially, the oil molar ratio of *C. vulgaris*, by varying the concentration of methanol and oil in the transesterification process. With an increasing trend, the yield of C. vulgaris biodiesel reached its maximum value at 87.6% for a molar ratio of 4:1, and remained constant beyond this mixing ratio. Supporting this, El-Batal et al. (2016) reported maximum biodiesel yield of 90% upon maintaining the molar ratio as 4:1, during the transesterification of waste cooking oil using the lipase isolated from *Aspergillus niger*, coated on barium ferrite magnetic nanoparticles was used as bio-nano catalyst. In fact, presence of excess methanol in the reaction system had no significant effect on the biodiesel yield; and this was in contrast to change in biodiesel yield for conventional acid/base catalyzed transesterification reactions. However, very slight change in the biodiesel yield was noted with the increase in methanol concentration in the reaction system. Lastly, the Volume of methanol (in ml) required for carrying out the transesterification was calculated based on the equation reported by Srinivasan et al. (2020).

3.8. Effect of temperature in enzyme transesterification

Temperature is one of the important parameter in process of enzymatic transesterification. When the bio-oil into FAME conversion, the effect of temperature was studied by varying different temperatures at 5 °C interval, wherein the range of temperature was varied between 15-55 °C, at 400 rpm with 4:1 methanol: oil ratio for about 6 h. In this study, highest biodiesel yield was noted as 88% (Fig. 6a), at 45 °C; beyond which it started reducing stating the denaturing of protein at higher temperatures reduced its catalytic effectivity, thereby resulting in poor catalytic activity and reduced FAME conversion.

3.9. Effect of catalyst concentration in enzymatic transesterification

The role of the catalyst was investigated in enzyme transesterification reaction by varying catalyst concentration ranges from 100–500 mg at constant temperature 45 °C and 400 rpm respectively for about 6 h (Fig. 6b). It was noted that the highest biodiesel yield (87.6%) was reported upon optimizing the catalyst concentration as 300mg; and was explained its sufficient availability, which in turn offered the availability of sufficient number of active sites to undergo effective conversion. However, increased concentration of catalyst simply resulted in outnumbering the active sites of the oil and methanol; instead resulted in the aggregation of the nanoparticles leading to formation of macro sized particles and reduced its catalytic activity. This phenomenal change simply led to reduced conversion rate and poor biodiesel yield (Kumari et al., 2009; Maceiras et al., 2009; Jegannathan et al., 2010).

3.10. Effect of reaction time in enzymatic transesterification

To understand the effect of time duration on the enzymatic transesterification, the reaction was carried out for varying reaction time durations by maintaining other reaction parameters like temperature, agitating speed and catalyst concentration as constant. Accordingly, the reaction time duration was varied between 2 and 10 h (Fig. 6c), and the optimized time duration was found to be 6 h, with highest reportable yield as 87.6%. This longer time durations are explained by the reduced reaction kinetics of enzymatic transesterification reaction than compared to conventional acid/base catalyzed reactions.

3.11. Effect of agitation in enzymatic transesterification

Of major concern, agitation speed is regarded as a vital parameter for any enzymatic transesterification reaction; and presently, the effect of agitation during the transesterification of C. vulgaris oil was studied by varying the speed between 100 and 500 rpm, in multiples of 100 rpm (Fig. 6d). Here, highest biodiesel yield was reported at 400 rpm and was calculated as 87.6%. In fact, the agitation helped in overcoming the mass transfer barrier between the reactants (oil and methanol) and helps in diffusing the reactants on to the external surface of the catalyst, then later on into their interior pores and active sites (Kumari et al., 2009). Besides, appropriate agitation of the reaction mixture favored the forward immobilization reaction.

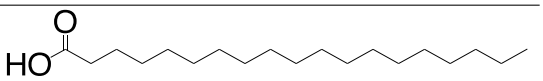
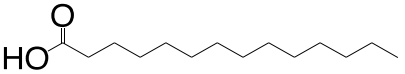
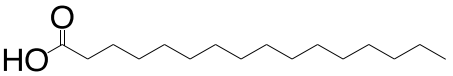
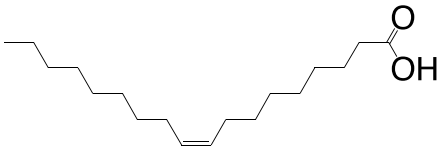
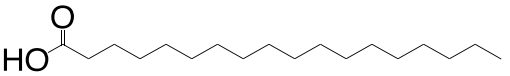
3.12. Biodiesel characterization study

To identify the fatty acid methyl ester conversion, the biodiesel produced from algal lipid of *Chlorella vulgaris* was analyzed using GC–MS in total scan mode. The individual peak identification was interpreted using the NIST inbuilt library, Vellore Institute of Technology, Tamil Nadu, India. After interpretation, five fatty acid methyl ester compounds were identified in the produced biodiesel; and are nonadecanoic acid methyl ester, tetradecanoic acid methyl ester, n-hexadecanoic acid methyl ester, oleic acid methyl ester, and 9-octadecanoic acid methyl ester. Based on the GC–MS results, the C. vulgaris lipid was distributed with both saturated and unsaturated fatty acids, and had a significant role in the biodiesel production. The FAME obtained from the lipids of *Chlorella vulgaris* characterized by GC–MS (SF. 1 and Table 1). After interpretation, the biodiesel from microalgal lipid reported its fuel properties similar to that of standard diesel. In 40 °C and 30 °C temperature, the viscosity of biodiesel was found at respective range of 3.421 mm ^{2}/sec and 0.87 g/cm^3 and it calculated after the interpretation with standard range. The microalgae extracted biodiesel can be used directly in the existing engines because of the low viscosity, and also slightly higher heating value. Besides that, the high percentage of oxygen content in the microalgae extracted biodiesel compared with conventional diesel helped it in achieving the complete combustion. Again, produced biodiesel reported higher flash point, thereby making it safer for storage and transportation. Next up, cetane number of the produced biodiesel was found as 60, which helped it in reducing its ignition delay, improvised its cold start behavior, and ensured smooth running of the engine (Srinivasan et al., 2020). Owing to increased concentration of unsaturated FAMEs, the resultant biodiesel reported its cold point temperature to be slightly closer to that of neat diesel, Johnson and Wen (2009) also said that the storage increase when add the stabilizer. Finally, we concluded that the range of 115 °C for flashpoint, −12 °C for pour point and 7 °C for cloud point of the sample were observed.

4. Conclusion

The conclusion drawn from the current experimental study suggests that the industrial waste water mediated green microalgal strain was found to be capable bioremediating the pollutant which present in the wastewater as well as effective enough to be used for biofuel production. In specific, this study reported that the identified strain reported high lipid content and this lipid was used for producing biodiesel using lipase immobilized magnetic nanoparticles. The biomass growth was high and lipid productivity was significant, when grown under mixotrophic conditions using sugar molasses (10 g L^{-1}) as a carbon respectively. On the other hand, the physical and chemical properties of the produced biodiesel, evaluated as ASTM standards, were in significant agreement with diesel. In the future, microalgae have the potential important in sustainable renewable energy feedstock and biofuel production from microalgae that could meet the global energy demand.

Table 1
Fatty acid profile of biodiesel from lipid of *Chlorella vulgaris*.

S.No	Retention time	Fatty acids	Structure
1	12.24	Nonadecanoic acid	
2	13.03	Tetradecanoic acid	
3	15.18	n-Hexadecanoic acid	
4	17.1	Oleic acid	
5	20.39	9- Octadecenoic acid	

CRediT authorship contribution statement

G. Rajivgandhi and J. Ranjitha designed and done most of the work. G. Ramachandran and M. Maruthupandy characterized the Oils related work. The entire lipid related work done by S. Vijayalakshmi and Chenthis Kanisha Chelliah. All the nanoparticles parts conducted by Franck Quero, Fahd A. AL-Mekhlafi, and Muhammad A. Wadaan also contributed written part of the manuscript. Wen-Jun Li contributed in the whole manuscript, structure, grammar mistakes and some additional experiments.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary material related to this article can be found online at https://doi.org/10.1016/j.eti.2022.102597.

References

Batal, A.I.E., Farrag, A.A., Elsayed, M.A., Khawaga, A.M.E., 2016. 'Biodiesel production by aspergillus niger lipase immobilized on barium ferrite magnetic nanoparticle'. Bioengineering 14, 212–218.
Elumalai, S., Prakasam, V., Selvarajan, R., 2011. 'Optimization of abiotic conditions suitable for the production of biodiesel from *Chlorella vulgaris*'. Indian J. Sci. Technol. 4 (2), 91–97.
Forján, E., Garbayo, I., Henriques, M., Rocha, J., Vega, J., Vílchez, C., 2011. UV-A mediated modulation of photosynthetic efficiency, xanthophyll cycle and fatty acid production of nannochloropsis. Mar. Biotechnol. 13, 366–375.
Hamedi, S., Mahdavi, M.A., Gheshlaghi, R., 2016. 'Improved lipid and biomass productivities in chlorella vulgaris by differing the inoculation medium from the production medium'. Biofuel Res. J. 10, 410–416.
Heredia, V., Gonçalves, O., Marchal, L., Pruvost, J., 2021. Producing energy-rich microalgae biomass for liquid biofuels: Influence of strain selection and culture conditions. Energies 14 (5), 1246.
Jambulingam, R., Shalma, M., Shankar, V., 2019. Biodiesel production using lipase immobilised functionalized magnetic nanocatalyst from oleaginous fungal lipid. J. Clean. Prod. 215, 245–258.
Jegannathan, K.R., Yee, L.J., Chan, E.S., Ravindra, P., 2010. 'Production of biodiesel from palm oil using liquid core lipase encapsulated in k-carrageenan'. Fuel 89, 2272–2277.
Johnson, M.B., Wen, Z., 2009. Production of biodiesel fuel from the microalga *Schizochytrium limacinum* by direct transesterification of algal biomass. Energy Fuels 23 (10), 5179–5183.

Kamyab, Hesam, Lee, Chew Tin, Din, Md, Fadhil, Mohd, Mohanadoss, Ponraj, Eva Mohamad, Eva, Mohamad, haza, Sohrabi, Mohsen, 2013. Effects of nitrogen source on enhancing growth conditions of green algae to produce higher lipid. Desalin. Water Treat. http://dx.doi.org/10.1080/19443994.2013.854030.

Kandasamy, R., Kennedy, L.J., Vidya, C., Boopathy, R., Sekaran, G., 2010. Immobilization of acidic lipase derived from pseudomonas gessardii onto mesoporous activated carbon for the hydrolysis of olive oil. J. Mol. Catal. B: Enzymatic 62 (1), 58–65.

Kumari, A., Mahapatra, P., Garlapati, K.V., Banerjee, R., 2009. 'Enzymatic transesterification of Jatropha oil'. Biotechnol. Biofuels 2 (1), 221–227.

Maceiras, R., Vega, M., Costa, C., Ramos, P., Marquez, M.C., 2009. 'Effect of methanol content on enzymatic production of biodiesel from waste frying oil'. Fuel 88, 2130–2134.

Matsunaga, T., Matsumoto, M., Maeda, Y., Sugiyama, H., Sato, R., Tanaka, T., 2009. Characterization of marine microalga, Scenedesmus sp. strain JPCC GA0024 toward biofuel production. Biotechnol. Lett. 31 (9), 1367–1372. http://dx.doi.org/10.1007/s10529-009-0029-y, Epub 2009 May 28.

Mohanraj, R., Mythili Gnanamangai, B., Rajivgandhi, G., Wen-Jun, Vijayalakshmi, G., Ponmurugan, P., Alharbi, N.S., Kadaikunnan, S., Khaled, J.M., Alanzi, K.F., 2021. Monitoring the decolourisation efficacy of advanced membrane fabricated phytosilica nanoparticles in textile effluent water treatment. Chemosphere 273, 129681.

Mostafa, S. Elshashed, 2010. Microbiological aspects of biofuel production: Current status and future directions. J. Adv. Res. 103–111.

Munir, M., Ahmad, M., Saeed, M., Waseem, A., Nizami, A.S., Sultana, S., Zafar, M., Rehan, M., Srinivasan, G.R., Ali, A.M., Ali, M.I., 2020. Biodiesel production from novel non-edible caper (Capparis spinosa L.) seeds oil employing Cu–Ni doped ZrO2 catalyst. Renew. Sustain. Energy Rev. 110558.

Raita, M., Arnthong, J., Champreda, V., Laosiripojana, N., 2015. 'Modification of magnetic nanoparticle lipase designs for biodiesel production from palm oil'. Fuel Process. Technol. 134, 189–197.

Rajivgandhi, G., Vimala, RTV, Nandhakumar, R., Murugan, S., Alharbi, N.S., Kadaikunnan, S., Khaled, J.M., Alanzi, K.F., Li, Wen-Jun, 2021. Adsorption of nickel ions from electroplating effluent by graphene oxide and reduced graphene oxide. Environ. Res. 199, 111322.

Shalini, P., Raman, L.A., Santhanakrishnan, S., Vijayalakshmi, S., Ranjitha, J., 2021. A review on recent trends in the microbial production of biodiesel. In: AIP Conference Proceedings Vol. 2396, no. 1. AIP Publishing LLC., 020007.

Siahmard, O.J., Pableo, R.M.B., Novero, A.U., 2017. Molecular identification of rhizospheric fungi associated with'Saba'banana via the amplification of internal transcribed spacer sequence of 5.8 S ribosomal DNA. Asian J. Plant Sci. 16 (2), 78–86.

Srinivasan, G.R., Shankar, V., Sekharan, S.Chandra., Munir, M., Balakrishnan, D., Mohanam, A., Jambulingam, R., 2020. Influence of fatty acid composition on process optimization and characteristics assessment of biodiesel produced from waste animal fat. Energy Sources A: Recovery Util. Environ. Effects 1–19.

Xie, W., Ma, N., 2010. 'Enzymatic transesterification of soybean oil by using immobilized lipase on magnetic nano-particles'. Biomass Bioenergy 34 (6), 890–896.

Yang, Z.K., Niu, Y.H., Ma, Xue, J., Zhang, M.H., Yang, W.D., Lu, S.H., Guan, H.Y., 2013. 'Molecular and cellular mechanisms of neutral lipid accumulation in diatom following nitrogen deprivation'. Biotechnol. Biofuels 6, 67–72.

Zhu, L., Gao, N., Cong, R.G., 2017. Application of biotechnology for the production of biomass-based fuels.