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Comparative Pretreatment Strategies for Efficient Lipid Extraction Followed by the Valorization of Algal Biomass for Biomaterial Production

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Abstract: Industrialization has resulted in a scarcity of energy reserves and an alarming exigency for sustainable fuels. The significant consumption of fossil fuels has caused global warming anddetrimental effects on the global economy. Photosynthetic microalgae can serve as both an alternative source of energy and a commercial product. Low lipid extraction yield significantly restricts microalgae from being a viable feedstock at a large scale. Hence, in this study, diverse traditional methods (TMs), including ultrasonication, osmotic shock, microwave, and acid-base hydrolysis, were investigated compared to a novel combinatorial method (NCM) developed for the pretreatment of microalgae biomass. NCM was chosen as the most appropriate strategy as it showcased a 2.2-fold rise in the total lipid yield in contrast with the considerable TMs. Additionally, the defatted algae biomass (DFB)was repurposed to synthesize commercially significant zinc oxide nanoparticles (ZnO, possessing an average size of 295 nm. The synthesized nanoparticles were analyzed by a suite of characterization methods likeScanning electron microscope (SEM), X-ray powder diffraction (XRD), and Dynamic light scattering (DLS) to comprehend the structural and morphological insights. Therefore, the unprecedented work demonstrated the microalgae biorefinery approach for biodiesel application and simultaneous production of ZnO nanoparticles to support a zero-waste approach.

Keywords: microalgae; pretreatment; lipid; biodiesel; defatted biomass; nanoparticles.

1. Introduction

Fossil fuels are considered the most essential resource for humankind, as they facilitate quotidian life. Nevertheless, in modern times, the necessity for anthropogenic choresand the growing population have resulted in extensive exploitation of non-sustainable feedstocks. Therefore, extensive research has been conducted to investigate the opportunities in renewable and carbon-neutral biofuels, as this strategic pathway would provide sustainable solutions toward greener production processes [1]. Oxygenous microalgae biomass is a dynamic feed that can be easily valorized via a wide variety of techniques, and it also possesses the ability to reduce the ecological footprint. Due to the ease of cultivation, high biomass yield, and utilization of waste as nutrients, algae are a reassuring alternative for biodiesel production [2]. However, the low lipid production is a significant impediment to the feasibility of microalgae-

based biodiesel [3]. The appropriate pretreatment of biomass is a prerequisite for obtaining the optimal recovery of biofuel precursors.

Conventionally, biomass pretreatment is performed primarily with pulsed electric fields, microwaves, solvents, and ultrasound [4]. However, it has been studied that these processes are costly and energy-intensive, and complete lipid recovery is not achieved [5]. As in a report on the pretreatment of *Chlorella homosphaera* biomass, it was concluded that the sequential procedure of chlorophyll removal and cell disruption can facilitate lipid extraction. However, the basis of specific energy input and scalability has not been identified [6]. In another study, the highest lipid yields (19.25%) were obtained by pretreatment of microalgae biomass withan ultrasonic homogenizer-assisted deep eutectic solvent [choline chloride: acetic acid (1:3)], followed by extraction over 120 min at 60°C [7].

In general, lipids are the most desired metabolites for extraction; nevertheless, carbohydrates and proteins play a substantial role in DFB. The extraction of various primary products from biomass, in addition to lipids, may aid in enhancing the financial sustainability of the microalgae sector. As a result, it would help transform the paradigm of biodiesel production from a solitary method to a holistic perspective [8]. In the past decade, nanotechnology has become a vital tool for recycling debris and synthesizing materials from sparse natural resources, supporting the concept of "green synthesis" as a path toward a more viable future [9]. For example, *Eucalyptus globulus* leaf extract was used to biosynthesize ZnO nanoparticles, but the land use for cultivating *Eucalyptus globulus* might make the process commercially unviable [10]. In another study, ZnO nanoparticles were derived from the biomass of *Lactobacillus plantarum*. It involved various steps, including several centrifugations and ultrasonication cycles, making the purification step very complex [11].

In the current investigation, two significant subjects of algal biorefinery were examined: firstly, developing a pretreatment method for efficient lipid extraction and secondly, repurposing thedefatted algal residue for synthesizing a nanomaterial. Most importantly, for the very first time, this combinatorial method has been developed and tested on *Asterarcys* sp. MRN2 is followed by the synthesis of ZnO nanoparticles, supporting the waste-free strategy.

2. Materials and Methods

2.1. Cultivation and harvesting of microalgae biomass.

The microalgae *Asterarcys* sp. MRN2 used in this study was isolated in our prior study, which is communicated [12]. Cells were cultivated under photoautotrophic culture conditions in 1000ml Erlenmeyer flasks containing 500 ml BG-11 medium [7]. Before cultivation, the culture medium was sterilized at 121°C for 20 min, and its pH value was adjusted to 7.2 ± 0.2. A photoperiod of 16 h light: 8h dark and a temperature of 25 ± 2°C for 30 days was maintained with intermittent shaking; no external CO_2 bubbling was supplemented to the cultivation system [13].

Algal biomass was harvested from the media by centrifugation at 3000 rpm for 10 min. The slurry obtained was lyophilized to determine the average moisture content of the harvested algal biomass. The lyophilized algal biomass was then adequately stored for later experimentation. The remaining centrifuged algal biomass was immediately preserved and centrifuged at -20°C.

2.2. Pretreatment of algal biomass.

2.2.1. Microwave.

The microwave treatment was carried out by subjecting 1 g of dried microalgae biomass at a power of 300 W for a variable duration of 5 min, 15 min, and 30 min. The protocol followed was the modification of a study by [14].

2.2.2. Ultra-sonication.

A homogenous mixture was prepared by dissolving 1g biomass in 5 ml dH_2O for ultrasonication. Further, the prepared mixture was treated in a sonicator bath at 27°C for 15, 30, and 45 minutes [14].

2.2.3. Acid-base hydrolysis.

Acid-base hydrolysis was performed by preparing 5 ml each of 1M sulphuric acid and sodium hydroxide solution. 1M H_2SO_4 was added dropwise to 1 g of algal biomass, and the mixture was kept in a hot air oven at 90°C. Furthermore, repeated heating was done by adding sodiumhydroxide. The mixture was repeatedly washed with dH2O by centrifuging at 3000 rpm for 3 minutes [15].

2.2.4. Osmotic shock.

The osmotic shock treatment was achieved by processing 1 g biomass with variable concentrations (0.5 M, 1 M, and 1.5 M) of sodium chloride solution each. The mixture waskept in an incubator shaker at 20°C and 80 rpm for 15 min [16].

2.2.5 Novel combinatorial methods (NCM).

On the basis of the different combinations, four different methodologies were developed in thecurrent study (Table 1). In all the methods, the harvested algae biomass was crushed into a finepowder treated with 1M H_2SO_4 at 90°C for 30 min to facilitate cell wall breakdown and release lipids. Subsequently, a base treatment was performed at 90°C for 30 min to neutralize the acidity and further enhance lipid extraction by administering the mixture with 1M NaOH. Ultrasonication was performed at 27°C for 30 min to disrupt cellular structures and promote the release of intracellular lipids. Lastly, the mixture was repeatedly washed with dH_2O by centrifuging at 3000 rpm for 3 min to remove impurities and separate the lipid-rich fraction.

Table 1. Stepwise methodology followed for the developed combinatorial techniques.

Novel Combinatorial Methods	Acid treatment at 90°C for 30 min	Base treatment at 90°C for 30 min	Ultrasonication at 27°C for 30 min	Acid treatment + Ultrasonication at 27°C for 30 min	Base treatment + Ultrasonication at 27°C for 30min
Method 1	Step 1	Step 2	Step 3	-	-
Method 2	Step 1	Step 3	Step 2	-	-
Method 3	Step 2	-	-	-	Step 1
Method 4	-	Step 2	-	Step 1	-

2.3. Lipid extraction and quantitation.

Following the various pretreatment procedures, the lipids were extracted and quantitated to infer the efficiency of the methods employed [13]. The gravimetric analysis was performed

using the Bligh and Dyer method [17], with the incorporation of chloroform and methanol (2:1) in 1g DCW. The lipid production was determined using Eqn. (1).

Lipid production (g/L) = mass of lipid (g)/mass of biomass (g) (1)

2.4. Conversion of defatted biomass to ZnO nanoparticles.

ZnO nanoparticle synthesis was carried out using zinc sulfate ($ZnSO_4$, Sigma Aldrich). The obtained defatted algae biomass was mixed with dH_2O and heated at 70°C for 30 min. The mixture was filtered through the muslin cloth and Whatman filter paper; the extract produced herewith synthesized ZnO nanoparticles. In the current study, 95 ml of 0.01 M zinc sulfate solution was added to 5 ml of algal extract; this mixture was incubated at 150 rpm with continuous shaking for 1 h at 70°C. The nanoparticle synthesis was confirmed by the appearance of the precipitates settled at the bottom of the flask. The collected precipitates were washed repeatedly with dH_2O to remove the impurities [18].

$ZnSO_4$ + Microalgae extract → ZnO-NPs + By-products (2)

2.5. Characterisation of the synthesized nanoparticles.

2.5.1. SEM and EDS analysis.

High-resolution SEM (SEM- SUPRA 40, Carl Zeiss, Germany) was used to examine the surface characteristics of the ZnO sample. Energy dispersive X-ray spectroscopy (EDS) was used to determine the elemental composition of the samples after they were sputtered with a gold coater at a voltage of 5 kV.

2.5.2. UV-Vis absorbance.

The light absorbance of the algae-derived nanoparticle was studied by a UV-Vis spectrophotometer (BioTek Instruments, Winooski, VT, USA). The absorption spectrum was recorded in the range between 280-800 nm.2.5.3 XRD.

Powder XRD (Bruker D2 Phaser, Massachusetts, USA) was used, with a voltage and current of 40 kV and 30 mA, respectively, in the scan range 2Θ = 20°-80°. Cu Kα1 radiation was usedto record the X-ray in a finely powdered solid sample.

2.5.3. DLS.

The DLS of the ZnO suspensions was measured in polystyrene cuvettes using a Zetasizer (Nano-S90, Malvern, UK). Measurements were taken of suspensions diluted suspensions to mitigate particle-to-particle interactions.

2.6. Statistical analysis.

All experiments were performed in triplicate, and the findings were calculated as mean and standard deviation. The data were analyzed using OriginPro 2021 (OriginLab, Northampton, MA, USA).

3. Results and Discussion

3.1. Pretreatment effect on microalgae lipid yield.

The *Asterarcys* sp. MRN2 cells were cultivated in an autotrophic mode for over 30 days to produce the biomass to be treated for lipid extraction. Pretreatment experimentswere carried out on the biomass harvested from the same cultivation slot. The lipid yield estimated for the control cells of *Asterarcys* sp. MRN2 in all experiments was 250±1.7 mg/g. Microwave treatment for 5 min at 300 W ensured the highest lipid yieldof 210 mg/g (Figure 1a), whereas ultrasonication at 27°C for 30 min resulted in the highest lipid production of 280 mg/g (Figure 1b).

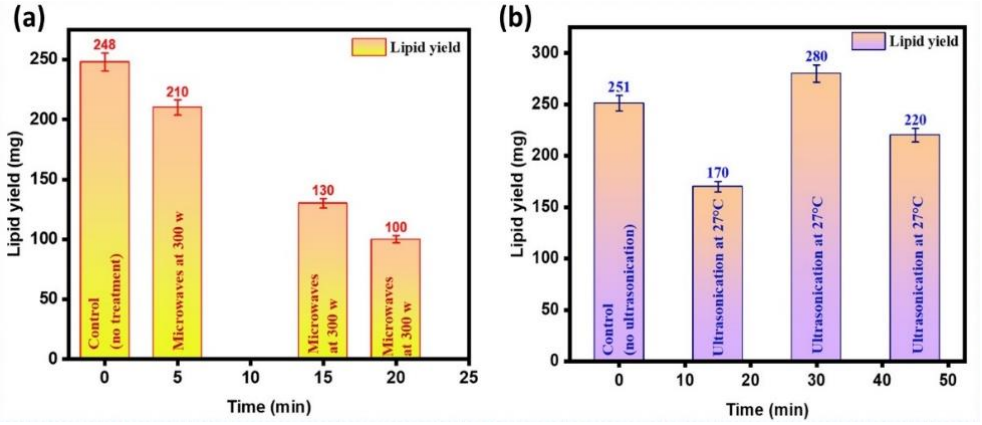

Figure 1. Estimated lipid yield of *Asterarcys* sp. MRN2 with (**a**) microwave treatment; (**b**) ultrasonication treatment for variable time duration.

Hence, unlike the microwave treatment, the lipid yield delivered by the best-optimized condition of ultrasonication was ~1-fold higher than that of control cells. However, microwave treatment was found to be responsible for poor lipid extraction efficiency; parallel results were observed in an investigation performed on *Tetraselmis suecica* [14].

The increase in lipid yield extraction following the ultrasonic pretreatment is attributed to the increase in surface area generated by microalgal cell rupture. This was mirrored inthe procedure, leading to breaking microalgal cells into smaller fragments over time. Acoustic cavities are formed during ultrasonic as a sufficiently powerful ultrasonic force propagates in a liquid. A large amount of energy is releasedwhen these cavitation bubbles collapse, forming high-amplitude shock waves. As a result,the microalgal cells burst due to external stresses. The degree of cell rupture and breakdown is determined by the degree of interaction between the cells and cavitation bubbles [19]. These findings were consistent with the available literature, which shows that ultrasonication can be an effective pretreatment strategy for increasing lipid recovery, and the ideal pretreatment procedure settings may vary based on the strain investigated [20].

Further, acid-base and osmotic shock pretreatment methods were investigated for lipid extraction efficiency. As shown in Figure 2a, after treating the algal cells with the acid-base combination, the highest yield of 333 mg/g was obtained from the cells kept in a hot air oven at 90°C for 30 min. This was ~ 1.3-fold more than the control cells.

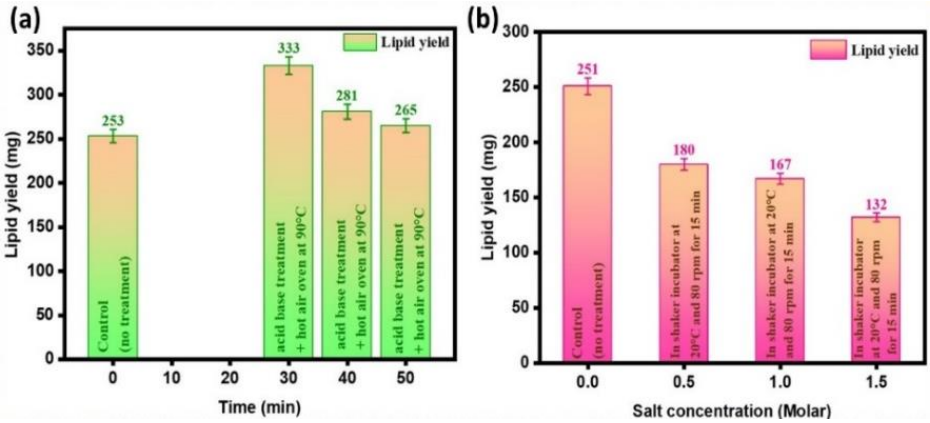

Figure 2. Estimated lipid yield of *Asterarcys* sp. MRN2 with **(a)** acid-base treatment for variable time duration; **(b)** osmotic shock treatment for different salt concentrations.

In contrast, the osmotic shock treatment (180 mg/g) resulted in considerably lower lipids than the control cells (Figure 2b). According to the aforementioned experimentation, acid-base hydrolysis followed by ultrasonication was found to be most competent for increasing the total lipid extraction.

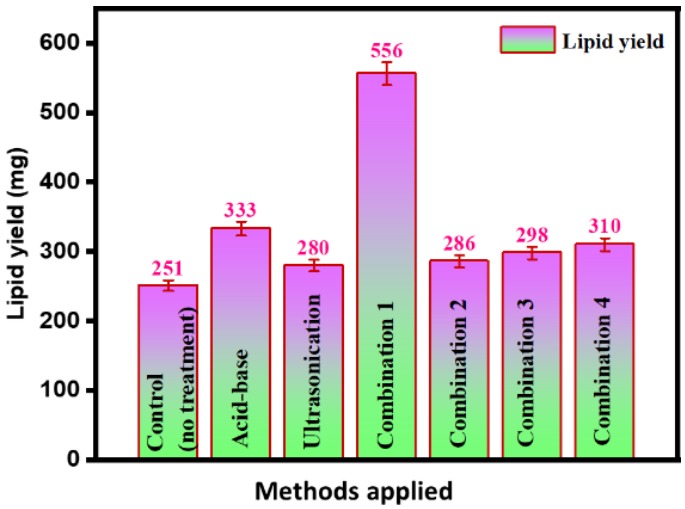

Figure 3. Comparative estimation of lipid extracted from *Asterarcys* sp. MRN2 control, optimized pretreatment by acid-base and ultrasonication; four novel combinations developed.

On the basis of the preliminary results, four NCMs were developed and investigated for their effect on the lipid yield (Figure 3). After the experimentation, combination 1 was acid-base treatment at 90°C for 30 min followed by ultrasonication at 27°C for 30 min, providing a lipid yield of 556 mg/g, which was the maximum among all the studies. Thisenormous boost in the recovery could be credited to the optimal collisions generated by the adequate supplementation of acid-base and ultrasonic waves. It could minimize the interaction of biopolymers in the cell wall as the ultrasonic waves formthe acoustic cavities.

Thus, cleaving some recalcitrant bonds makes the cell wall vulnerable to acid-base attack, enhancing lipid yield. However, hydroxyl ions in the algal suspension might get through the cell wall and cell membrane, reacting with the cellular lipid to produce glycerol and fatty acid salts. As glycerol is insoluble in chloroform, only fatty acids were extracted by chloroform after acidifying the suspension [21]. In a recent study on *Chlorella pyrenoidosa*, the impact of Ultrasonic- alkali pretreatment was found effective for microalgae acidification; the pretreatment procedure also reported an increase in the volatile fatty acids [22]. Thus, the current study is the first report to explore the combination involving both acid-base and ultrasonicationtreatment simultaneously.

3.2. Synthesis and characterization of ZnO nanoparticles from defatted residue.

The DFB of *Asterarcys* sp. MRN2 obtained from the foregoing experiments was successfully employed to produce ZnO nanoparticles. The detailed functional and structural characterization techniques confirmed the synthesis of the stated nanoparticles. SEM and EDS analysis.

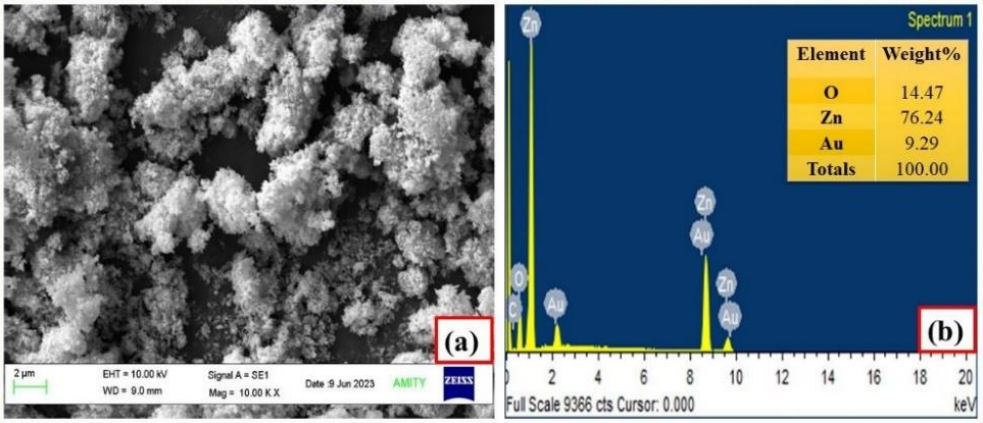

Figure 4. (a) SEM; **(b)** EDS image of the ZnO nanoparticles synthesized from the DFB of *Asterarcys* sp. MRN2.

SEM was done to study the surface morphology of nanoparticles. It can be observed from Fig.4a that the morphology of zinc oxide nanoparticles is spherical, evenly dispersed, and aggregated. The aggregation can be either due to the maximum surfaceenergy of ZnO-NPs that often happens during the preparation of aqueous media or because of the surface area to volume ratio of spherical nanoparticles [23].

EDS analysis was used to examine and increase the secondary vision into the topographies of nanoparticles. In Fig.4b, two sharp peaks for zinc around 1 keV and 8.7 keV and a singular peak for oxygen at ~ 0.5 keV validate the presence of typical zinc in the oxide form [10]. The peak spectrum displays zinc and oxygen emission energies, which have been confirmed by existing studies [24].

3.2.1. XRD.

The crystallographic structure of nanoparticles and uniformly arranged atomic structure were analyzed using the XRD technique. All the peaks were assembled with the ZnO hexagonal phase in accordance with the JCPDS file No. 36-1451 [25].

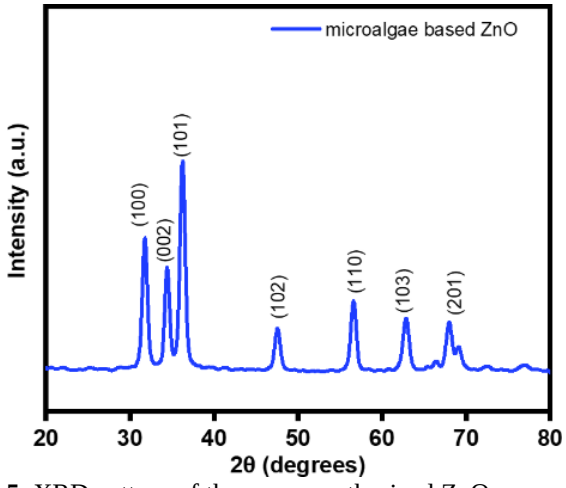

Figure 5. XRD pattern of the green synthesized ZnO nanoparticles.

The sharp and narrow diffraction peaks demonstrated the crystallinity and purity of the as-synthesizednanoparticles. Peaks were discovered at lattice planes (100), (002), (101), (102), (110), (103), and (201) in the corresponding zones of 31.7°, 34.4°, 36.2°, 47.4°, 56.5°, 62.8°, and 67.9° that correspond to those identified in hexagonal phase zincite [10].

3.2.2. UV-Vis absorbance and DLS.

UV–vis spectroscopy was performed at room temperature in a wavelength range of 230-800 nm to explore the optical characteristics of nanoparticles. As shown in Figure 6a, ZnO exhibited a distinct absorption peak between 360 and 370 nm [26]. The peak in the absorption spectrum can be attributed to the reduction of zinc ions bythe secondary metabolites present in the algal extract [27]. The surface plasmon absorption properties of ZnO nanoparticles lead to the transition of electrons from thevalence band to the conduction band. This phenomenon occurs when electromagnetic waves interact with surface plasmon absorption through cumulative oscillations of theopen conduction band electrons [28].

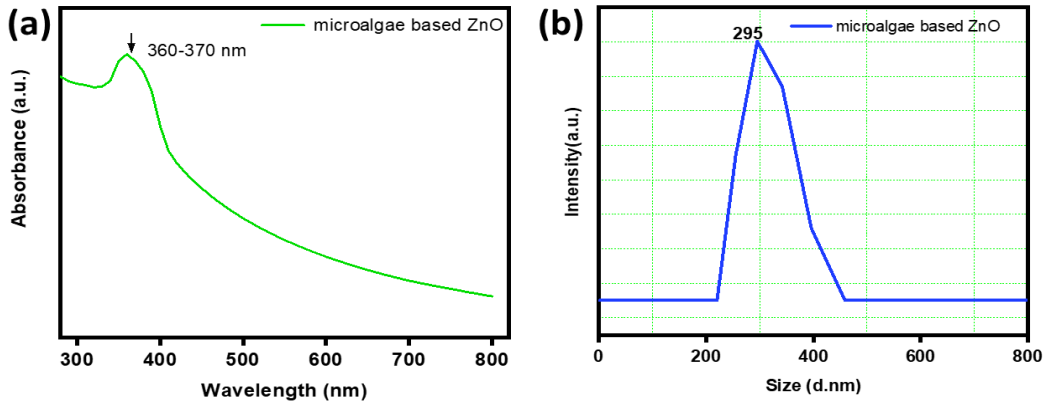

Figure 6. Characterization of ZnO nanoparticles **(a)** UV-Vis absorption; **(b)** DLS.

In order to obtain a reliable size measurement of NPs, regardless of size and shape, DLS or transmission electron microscopy (TEM) should be used. This study assessed the average particle size of biosynthesized ZnO NPs using the DLS method. The Z- average size of ZnO nanoparticles was estimated to be ~ 295 nm. The hydrodynamic size of the biosynthesized ZnO NPs was enormous due to measurements obtained from the metal core to the biological compound bonded to the particle surface, reflecting thehuge size [11].

4. Future perspectives and conclusions

The current study opens up disparate opportunities for future research and development in microalgae-based biorefinery. Firstly, optimization of the combinatorial technique should be investigated to maximize lipid yield while ensuring its scalability for large-scale production. This could entail experimenting with time, temperature,and chemical compositions in order to improve the efficacy of lipid extraction from microalgaebiomass. Nevertheless, producing ZnO nanoparticles from DFB presents new nanotechnology and materials research avenues. ZnO nanoparticles possess a wide range of applications, i.e., in agriculture, photocatalysis, medicine, food packaging, cosmetics, antioxidant prevention, anticancer drug delivery systems, and other activities related to their antimicrobial and antibacterial properties. The characterization techniques employedin this study provided vital insights into the structural and morphological features of the as-synthesized nanoparticle. Future research could strengthen the

synthesis method to increase nanoparticle yield, size homogeneity, and stability for use in catalysis, sensors, and optoelectronics.

In addition, an extensive economic and environmental assessment is required to examine the viability and sustainability of microalgae biorefinery. A life cycle assessment should be done to evaluate the entire process's environmental implications. Economic evaluations should consider issues such as manufacturing costs, market demand, and possible revenue streams from commercial microalgae products.

In conclusion, the study demonstrates the potential of microalgae as a useful resource for both energy production and commercial product synthesis. The novel combinatorial technique boosts lipid yield dramatically, making microalgae a feasible feedstock on a wide scale. Furthermore, a very economical method of synthesizing ZnO nanoparticles by using DFB wasexplored. These findings pave the way for additional research and development in the field of microalgae-based biorefinery, including prospects for optimization, nanotechnology applications, and complete economic and environmental sustainability analyses.

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Conflicts of Interest

The authors declare no conflict of interest.

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