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Integrated Stress-Induced Lipid Enhancement and Supercritical CO_2 Extraction for Efficient Biodiesel Production from Chlorella vulgaris

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ABSTRACT

This study investigates the effectiveness of supercritical carbon dioxide ($SC-CO_2$) extraction enhanced with n-hexane and ethanol co-solvents for recovering lipids from Chlorella vulgaris cultivated under stress conditions for biodiesel production. The microalgae were grown using a two-stage cultivation approach and categorized into three groups: a control (pre-stress), Treatment 1 (nutrient deprivation, pH 11, and 2 M NaCl for 24 h), and Treatment 2 (nutrient deprivation and 2 M NaCl for 72 h). Lipid extraction was carried out at pressures of 250, 300, and 350 bar and temperatures of 50 and 70 °C.

The total lipid contents obtained from the control, Treatment 1, and Treatment 2 groups were 8.78 ± 0.45%, 57.4 ± 0.75%, and 55.3 ± 1.03%, respectively, confirming substantial lipid accumulation under stress. The highest fatty acid yield (>99%) was consistently achieved at 350 bar and 50 °C for all groups. Furthermore, the physicochemical properties of the resulting biodiesel met both ASTM and EN standards.

Overall, the results demonstrate that combining stress-induced lipid enhancement with $SC-CO_2$ extraction offers an efficient and high-quality pathway for biodiesel production from Chlorella vulgaris.

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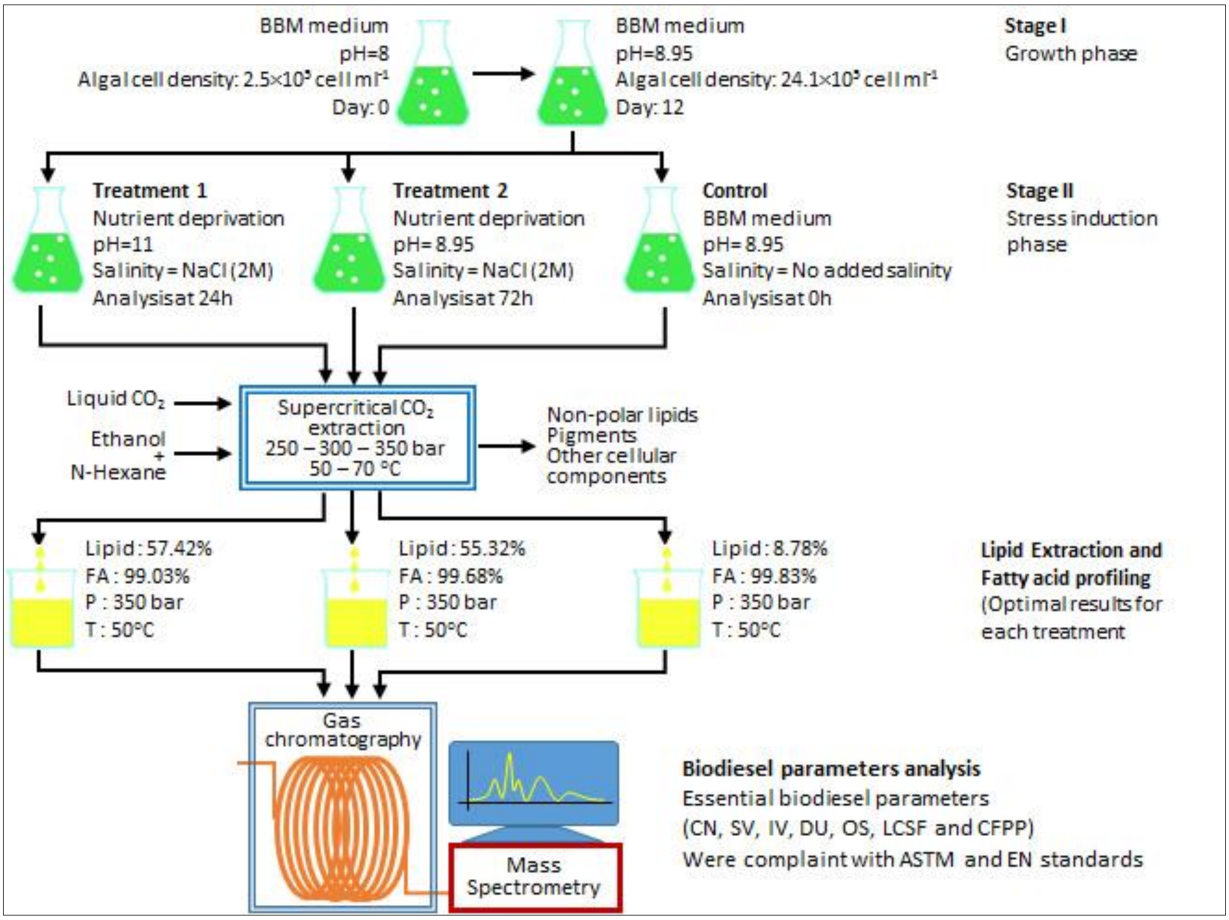

Introduction

With the development of human societies and the growing global demand for energy, the combustion of various materials—particularly fossil fuels such as oil, coal, and natural gas—has significantly increased. The sharp rise in oil prices during the 1970s motivated the search for alternative fuels, as the control of fuel prices in different countries became a matter of national security [1]. The increasing cost of fossil fuels has inevitably reduced their consumption, and their long-term economic feasibility is expected to diminish in the future [2]. Therefore, second-generation biofuels (derived from agricultural waste and animal biomass) and third-generation biofuels (derived from algae) are among the most promising alternatives to fossil fuels for future energy systems [1-3].

The International Energy Agency has predicted that the global consumption of biofuels for transportation will increase from 2% in 2011 to 27% by 2050, significantly reducing carbon dioxide emissions [4]. In 2009, the annual production of extractable oils and biofuels reached approximately 12,150 million tons, supplying 25% of transportation fuel demand [5].

Biodiesel, a renewable biofuel, is produced through transesterification reactions between fatty acids and alcohols. Fatty acids can be obtained from a broad range of feedstocks [3]. Animal fats, vegetable oils, and waste cooking oils are commonly used as primary biofuel sources [1-6-7]. Third-generation or advanced biofuels refer to those produced from algae [8]. Unlike first-generation biomass, algal feedstock has minimal or no impact on food supply or land use and exhibits high productivity [9-10].

The ability of microalgae to accumulate large amounts of lipids under controlled cultivation has attracted increasing scientific and industrial attention over the past two decades [11]. Algae require light, nutrients, carbon dioxide, and water for their rapid growth and photosynthetic activity [12]. *Chlorella vulgaris* is a unicellular microalga capable of storing valuable amounts of lipids suitable for biodiesel production due to its advantageous fatty acid profile [13-14]. Its high growth rate and potential to produce substantial quantities of oil (8–36 times greater than oil palm per hectare annually) are among its most notable characteristics [15]. Under optimal conditions, *C. vulgaris* can replicate multiple times within 24 hours through autosporulation [16].

Various methods—including mechanical pressing, solvent extraction, microwave-assisted extraction, and supercritical fluid extraction—have been utilized for lipid extraction from microalgae [17-18]. Among these techniques, supercritical CO_2 extraction modified with ethanol or hexane is considered particularly efficient

[19] (Fig. 1). Organic solvents and supercritical CO_2 are widely used for industrial oil extraction; introducing the liquid phase into the solid phase facilitates lipid separation into the liquid phase [20]. Supercritical CO_2 extraction is regarded as a safe technology and a viable alternative to conventional organic solvent extraction, which can be toxic and lead to the formation of undesirable compounds [21]. Additionally, the method offers advantages such as low viscosity, high penetration ability, and easy solvent separation [22]. CO_2 is non-flammable, environmentally friendly, selective, and considered safe, with mild critical parameters (31 °C and 7.38 MPa) [23]. Its oxygen-free nature prevents oxidation, and the low operating temperature minimizes thermal degradation of sensitive compounds [22].

Supercritical CO_2 has demonstrated high extraction efficiency and can extract up to 97% of neutral lipids from *C. vulgaris* under optimized conditions using methanol as a co-solvent [24]. Kwan et al. (2016) [25] reported that this method reduces energy consumption and downstream processing costs (e.g., distillation). Studies have shown that the biodiesel conversion rate for algal lipids is 35% higher than that of animal fat (sheep) under similar extraction conditions [26]. Compared with organic solvents, this method exhibits lower toxicity and does not require liquid-solvent separation. Furthermore, methanol concentration significantly affects lipid extraction efficiency [27]. Under optimal conditions, SC-CO_2 yields lipids with higher neutral lipid content and lower phospholipid levels [28].

Supercritical CO_2 is a relatively good solvent for non-polar compounds such as triglycerides but does not dissolve phospholipids [21]. Compared with organic solvents, it also covers a broader range of extractable materials with less adverse impact on extraction processes [17]. Therefore, increasing pressure and temperature, along with appropriate biomass loading, enhance extraction efficiency and biodiesel production performance in SC-CO_2 systems [29].

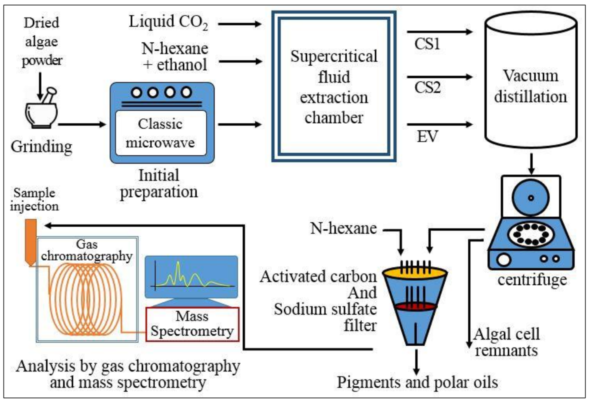

Figure 1. Schematic representation of the supercritical carbon dioxide (SC-CO_2) extraction process

Several studies have investigated methods and cultivation conditions to enhance lipid and fatty acid production in *C. vulgaris*, as well as various extraction techniques for biodiesel production. However, the combined effects of nutrient deprivation, 2-M NaCl salinity stress, pH = 11, and SC-CO_2 extraction at 250, 300, and 350 bar at 50 and 70 °C have not yet been evaluated.

Despite extensive research, the combined effects of nutrient deprivation, high salinity (2 M NaCl), alkaline conditions (pH 11), and supercritical CO_2 extraction under varying pressure and temperature conditions have not been fully investigated. Therefore, this study aims to evaluate these combined effects on lipid yield and biodiesel quality.

Materials and Methods

Microalgal Strain and Cultivation Conditions

A pure culture of *Chlorella vulgaris* (TAGACC004) was obtained from TAG Biotech Company (Tehran, Iran). The microalgae were cultivated using a two-stage strategy in 10-L flasks containing Bold's Basal Medium (BBM) at pH 8.0 and a temperature of 21 ± 1 °C. The cultures were exposed to cool-white fluorescent light (3000–3500 lux) under a 12:12 h light–dark cycle and continuously aerated at a rate of 800 mL/min.

After 12 days, the biomass reached its maximum cell density and was harvested by centrifugation at 3500 rpm for 5 min. The collected biomass was divided into three groups for stress induction.

Table 1. Induced Stress Conditions Applied to Experimental Treatments

Treatment	Description	Salinity Level	pH	Sampling & Oil Extraction Time
Control	Pre-stress	No added salinity	8.95	Prior to stress induction
Treatment 1	Nutrient deprivation + salinity + acidity	2 M NaCl	11	24 h after stress induction
Treatment 2	Nutrient deprivation + salinity	2 M NaCl	8.95	72 h after stress induction

Supercritical CO_2 Extraction

System Setup and Instrumentation

Supercritical fluid extraction was carried out using a TharSFC SFE-2000 system. The extraction was performed at pressures of 250–350 bar and

temperatures of 50–70 °C. A co-solvent mixture of n-hexane and ethanol (1:1, v/v) was used to enhance lipid recovery.
Each extraction run lasted 60 minutes. The CO_2 flow rate was initially set at 200 g/min and gradually reduced to 100 g/min, which was maintained until the end of the process.

Extraction Procedure

Supercritical CO_2 was used as the extraction fluid at temperatures of 50 and 70 °C and operating pressures of 250, 300, and 350 bar. A co-solvent mixture of n-hexane and ethanol (1:1 v/v) was employed to enhance extraction efficiency. The extraction time was 60 minutes. CO_2 flow rate was initially set at 200 g/min, gradually reduced to 100 g/min, and maintained at this value until the end of the process.
Following extraction, purification of samples was performed using n-hexane, sodium sulfate as a drying agent, and subsequently activated carbon filtration through a 1.2-µm GFC filter. A total of 30 g of dried microalgal powder was used for each extraction [22].
To prepare dried biomass, harvested algal slurry from stage two was filtered through Whatman filter paper and oven-dried at 54 °C for 24 h using a forced-air oven.
Fatty Acid Profiling
The extracted algal oil was injected into a gas chromatograph for fatty acid methyl ester (FAME) analysis, and the fatty acid composition was determined accordingly.
Assessment of Biodiesel Fuel Properties
Key biodiesel quality parameters—including saponification value, iodine value, degree of unsaturation, cetane number, long-chain saturated fatty acid (LCSF) content, and cold filter plugging point (CFPP)—were calculated using the following equations [30]:
Saponification value (SV):

$$SV = \frac{\sum(560 \times N)}{M}$$

Where N = percentage of each fatty acid, M = molecular weight of total lipids.
Iodine value (IV):

$$IV = \frac{\sum(254 \times D \times N)}{M}$$

Where N = percentage of each fatty acid, M = molecular weight of total lipids, D = number of double bonds.
Degree of unsaturation (DU):

$$DU = MUFA + (2 \times PUFA)$$

Cetane number (CN):

$$\mathrm{CN} = 46.3 + \frac{5458}{\mathrm{SV}} - (0.225 \times \mathrm{IV})$$

Long-chain saturated fatty acids (LCSF):

$$\mathrm{LCSF} = (0.1 \times \mathrm{C16}) + (0.5 \times \mathrm{C18}) + (1 \times \mathrm{C20}) + (1.5 \times \mathrm{C22}) + (2 \times \mathrm{C24})$$

Cold filter plugging point (CFPP):

$$\mathrm{CFPP} = (3.1417 \times \mathrm{LCSF}) - 16.477$$

Statistical Analysis

All data were first tested for normality using the Kolmogorov–Smirnov and Bartlett's tests. Factorial ANOVA was performed for samples analyzed at 24 h post-stress. ANCOVA was applied to the 24- and 72-h samples to eliminate the effects of prior time points, allowing evaluation of stress-induced changes independent of temporal variation.
One-way ANOVA was employed to compare mean values at each sampling time and to evaluate biodiesel parameters. Biodiesel fuel properties were further analyzed using ANCOVA relative to the baseline sampling time. A significance level of $p \leq 0.05$ was considered for one-way ANOVA, and $p \leq 0.001$ for factorial and ANCOVA tests. Statistical analyses were conducted using SPSS software (version 26).
To assess the interactive effects of algal treatments, pressure, and temperature on lipid content and fatty acid profiles, a 2×3×3 factorial design (18 treatments) was used and analyzed via two-way ANOVA at $p \leq 0.05$

Results

Effect of Stress Induction on Lipid Content of *Chlorella vulgaris*
As illustrated in Figure 2, the highest lipid content (57.42%) was obtained in Treatment 1 ($P \leq 0.05$).

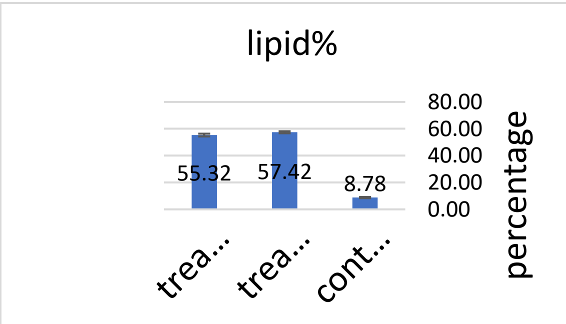

Figure 2. Lipid content of *Chlorella vulgaris* before and after the induction of physicochemical stress.

Effect of Stress Induction on Biodiesel Quality Parameters
As shown in Table 2, biodiesel quality indices—including fatty acid percentage(FA%), saponification value(SV), iodine value(IV) and cetane number (CN)—did not exhibit significant differences among treatments ($P \leq 0.05$).

Table 2. Biodiesel Quality Parameters of Chlorella vulgaris Oil

Treatment	Extraction conditions		Biodiesel Quality Parameters			
			FA%	SV	IV	CN
Control	250 bar	50 °C	$95.01^{c-f}\pm2.76$	$201.34^{a}\pm10.07$	$53.82^{d}\pm2.69$	$61.30^{ab}\pm3.07$
		70 °C	$79.92^{a}\pm3.60$	$203.20^{a}\pm9.15$	$43.05^{ab}\pm1.94$	$63.47^{ab}\pm2.86$
	300 bar	50 °C	$76.84^{f}\pm4.23$	$202.74^{a}\pm11.15$	$45.87^{cd}\pm2.52$	$62.90^{ab}\pm3.46$
		70 °C	$99.10^{ab}\pm0.46$	$201.35^{a}\pm9.06$	$54.42^{a}\pm2.45$	$61.16^{b}\pm2.76$
	350 bar	50 °C	$99.83^{ef}\pm0.09$	$201.26^{a}\pm12.08$	$52.98^{d}\pm3.18$	$61.50^{ab}\pm3.69$
		70 °C	$78.62^{a}\pm2.75$	$203.52^{a}\pm7.13$	$42.63^{a}\pm1.49$	$63.53^{b}\pm2.23$
Treatment 1	250 bar	50 °C	$93.76^{c-f}\pm4.69$	$204.84^{a}\pm10.24$	$48.18^{bc}\pm2.41$	$62.10^{ab}\pm3.11$
		70 °C	$90.33^{c}\pm4.07$	$203.07^{a}\pm9.14$	$51.77^{d}\pm2.33$	$61.53^{ab}\pm2.77$
	300 bar	50 °C	$89.44^{ef}\pm4.92$	$202.19^{a}\pm11.13$	$53.69^{b-d}\pm2.95$	$61.21^{ab}\pm3.37$
		70 °C	$98.81^{cd}\pm1.45$	$204.08^{a}\pm9.19$	$52.46^{cd}\pm2.36$	$61.24^{ab}\pm2.76$
	350 bar	50 °C	$99.03^{d-f}\pm0.95$	$204.27^{a}\pm12.26$	$50.50^{cd}\pm3.03$	$61.66^{ab}\pm3.70$
		70 °C	$90.78^{c-e}\pm3.18$	$202.66^{a}\pm7.10$	$53.15^{cd}\pm1.86$	$61.27^{ab}\pm2.15$
Treatment 2	250 bar	50 °C	$94.19^{c-f}\pm4.71$	$205.24^{a}\pm10.26$	$65.63^{e}\pm3.29$	$58.13^{ab}\pm2.91$
		70 °C	$93.27^{bc}\pm4.20$	$202.27^{a}\pm9.11$	$70.90^{ef}\pm3.19$	$57.33^{ab}\pm2.58$
	300 bar	50 °C	$87.11^{f}\pm4.79$	$202.86^{a}\pm11.16$	$69.08^{ef}\pm3.80$	$57.66^{ab}\pm3.17$
		70 °C	$99.42^{c-f}\pm0.18$	$204.74^{a}\pm9.22$	$69.04^{f}\pm3.11$	$57.42^{a}\pm2.59$
	350 bar	50 °C	$99.68^{ef}\pm0.28$	$204.73^{a}\pm12.29$	$67.40^{ef}\pm4.05$	$57.79^{a}\pm3.47$
		70 °C	88.59e±3.10	$203.66^{a}\pm7.13$	$67.92^{ef}\pm2.38$	$57.82^{ab}\pm2.03$

The superscript letters indicate non-significant differences at P < 0.05.

Furthermore, Table 3 indicates the degree of unsaturation (DU), oxidative stability (OS), long-chain saturated fatty acids (LCSF), and cold filter plugging point (CFPP).

Table 3. Biodiesel Quality Parameters: Degree of Unsaturation, Oxidative Stability, LCSF and CFPP

Treatment	Extraction conditions		Biodiesel Quality Parameters			
			DU	OS	LCSF	CFPP
Control	250 bar	50 °C	$35.90^{d}\pm1.80$	$3.19^{a-c}\pm0.16$	$0.25^{e-g}\pm0.01$	$-15.69^{a}\pm0.79$
		70 °C	$23.77^{a}\pm1.07$	$4.23^{d}\pm0.19$	$0.21^{a}\pm0.01$	$-15.82^{a}\pm0.71$
	300 bar	50 °C	$24.24^{d}\pm1.34$	$3.56^{bc}\pm0.20$	$0.20^{g}\pm0.01$	$-15.86^{a}\pm0.88$
		70 °C	$37.85^{a}\pm1.70$	$3.09^{e}\pm0.14$	$0.27^{ab}\pm0.01$	$-15.63^{a}\pm0.71$
	350 bar	50 °C	$37.65^{d}\pm2.26$	$3.26^{a-c}\pm0.20$	$0.27^{g}\pm0.02$	$-15.64^{a}\pm0.94$
		70 °C	$23.02^{a}\pm0.81$	$3.62^{d}\pm0.13$	$0.21^{ab}\pm0.01$	$-15.81^{a}\pm0.56$
Treatment 1	250 bar	50 °C	$27.89^{b}\pm1.40$	$3.36^{cd}\pm0.17$	$0.24^{d-f}\pm0.01$	$-15.72^{a}\pm0.79$
		70 °C	$31.10^{c}\pm1.40$	$3.20^{a-c}\pm0.15$	$0.23^{c-e}\pm0.01$	$-15.74^{a}\pm0.71$
	300 bar	50 °C	$32.63^{c}\pm1.80$	$3.16^{a-c}\pm0.18$	$0.23^{fg}\pm0.02$	$-15.75^{a}\pm0.87$
		70 °C	$32.97^{c}\pm1.49$	$3.16^{a-c}\pm0.14$	$0.26^{b-e}\pm0.02$	$-15.67^{a}\pm0.71$
	350 bar	50 °C	$31.68^{c}\pm1.90$	$3.19^{a-c}\pm0.20$	$0.26^{fg}\pm0.02$	$-15.67^{a}\pm0.94$
		70 °C	$32.85^{c}\pm1.15$	$3.19^{a-c}\pm0.11$	$0.24^{d-f}\pm0.01$	$-15.74^{a}\pm0.55$
Treatment 2	250 bar	50 °C	$36.99^{d}\pm1.85$	$3.03^{ab}\pm0.15$	$0.21^{a-c}\pm0.02$	$-15.80^{a}\pm0.79$
		70 °C	$43.48^{de}\pm1.96$	$2.94^{ab}\pm0.13$	$0.22^{a}\pm0.01$	$-15.79^{a}\pm0.72$
	300 bar	50 °C	$38.81^{ef}\pm2.14$	$2.98^{ab}\pm0.17$	$0.20^{b-e}\pm0.01$	$-15.85^{a}\pm0.87$
		70 °C	$41.98^{f}\pm1.89$	$2.95^{a}\pm0.13$	$0.23^{a-d}\pm0.01$	$-15.76^{a}\pm0.71$
	350 bar	50 °C	$41.13^{f}\pm2.47$	$2.96^{ab}\pm0.18$	$0.23^{b-e}\pm0.01$	$-15.76^{a}\pm0.95$
		70 °C	$38.77^{d-e}\pm1.36$	$3.00^{ab}\pm0.11$	$0.20^{a}\pm0.01$	$-15.84^{a}\pm0.56$

The superscript letters indicate non-significant differences at P < 0.05.

Discussion

The significant increase in lipid content under stress conditions can be attributed to metabolic shifts in microalgal cells. Nutrient deprivation, particularly nitrogen limitation, reduces protein synthesis and redirects carbon flux toward lipid biosynthesis. This process leads to the accumulation of acetyl-CoA, which serves as a precursor for fatty acid synthesis.

Additionally, high salinity and alkaline conditions may induce oxidative stress, further promoting lipid accumulation as a protective mechanism. These findings are consistent with previous studies reporting enhanced lipid production under stress conditions.

Under nitrogen stress conditions, *Chlorella vulgaris* undergoes significant metabolic reprogramming that shifts carbon flux from growth toward the accumulation of energy-dense storage molecules, particularly triacylglycerols (TAGs) [31-32]. This stress-induced lipid accumulation is mediated through a well-coordinated two-stage response. In the initial phase, nitrogen deprivation triggers the upregulation of genes involved in carbon fixation (RuBisCO, PEPCase), plastid protein degradation (CD4A), and starch synthesis (SS, SBE2) and leading to rapid carbohydrate accumulation [33-34]. During this stage, nitrogen assimilation-related genes are activated as a compensatory mechanism [31]. The subsequent stage is characterized by the upregulation of genes responsible for starch degradation (SP), glycolysis (PFO, PK, ACS), fatty acid biosynthesis (FabH, accD), and TAG assembly (DGAT2), resulting in the redirection of carbon toward lipid synthesis [33]. Acetyl-CoA, the essential two-carbon building block for fatty acid synthesis, is primarily generated via the pyruvate:ferredoxin oxidoreductase (PFO) reaction as an alternative to the conventional pyruvate dehydrogenase (PDH) pathway under stress conditions [32]. Additionally, the malic enzyme (ME) has been identified as the main source of NADPH—the key reducing equivalent required for fatty acid biosynthesis—in nitrogen-starved *C. vulgaris* cells [32]. The expression of the plastid-encoded β-carboxyltransferase subunit of acetyl-CoA carboxylase (accD), the rate-limiting enzyme in fatty acid synthesis, increases significantly under low nitrogen concentrations, further enhancing lipid biosynthesis capacity [33]. This comprehensive metabolic shift enables *C. vulgaris* to accumulate lipids exceeding 30–50% of its dry cell weight under optimal nitrogen starvation conditions, with saturated fatty acids (predominantly palmitic acid C16:0 and stearic acid C18:0) comprising the majority of the total fatty acid profile, making it a promising feedstock for biodiesel production [34-35] .

Effect of Physicochemical Stress on Lipid Accumulation in *Chlorella vulgaris*

In the present investigation, the lipid contents of the control culture, Treatment 1, and Treatment 2 were 8.75%, 57.42%, and 55.32% of the total biomass, respectively (Figure 1). These results provide compelling evidence that the application of physicochemical stress significantly enhances lipid biosynthesis in *Chlorella vulgaris*. Furthermore, the findings indicate that the combined stress condition—comprising nutrient deprivation, 2 M sodium chloride salinity, and an alkaline environment (pH 11)—was the most effective regime for maximizing lipid accumulation.

Effect of Stress Induction on the lipid Composition of *Chlorella vulgaris*

The findings of this study demonstrated that the application of various stressors positively influenced the lipid content of *Chlorella vulgaris*. Among all treatments, Treatment 1—comprising nutrient deprivation, alkaline pH (pH = 11), and 2 M NaCl salinity—resulted in the highest lipid yield. In agreement with these results, Adamakis et al. (2018) reported that elevated nutrient levels, particularly nitrogen, markedly suppress lipid accumulation in *C. vulgaris*. [36] Likewise, Ratomski and Hawrot-Paw (2021) observed that lipid production increases as nitrogen concentrations decrease [37].

Extensive research has confirmed that nitrogen limitation and reduced nitrate availability in the growth medium represent some of the most effective stimuli for inducing cellular stress and enhancing lipid biosynthesis in microalgae [13-38-39]. Therefore, decreasing nitrogen availability can be considered a reliable strategy for promoting lipid accumulation in algal biomass.

From a photosynthetic and metabolic perspective, nutrient deprivation—particularly nitrate limitation—reduces the cellular consumption of NADPH, resulting in its intracellular accumulation. This process inhibits the amino acid synthesis pathway and ultimately decreases algal protein content [40]. Under such conditions, acetyl-CoA is unable to enter the citric acid cycle because high NADPH levels inhibit citrate synthase activity, leading to further accumulation of acetyl-CoA. The excess acetyl-CoA can subsequently be converted to malonyl-CoA by acetyl-CoA carboxylase, which then serves as the primary carbon source for fatty acid biosynthesis [31].

Biodiesel quality indices

The iodine value of *Chlorella vulgaris* samples differed significantly among treatments as pressure increased, with Treatment 2 exhibiting the highest values. Iodine value is an indicator of overall biodiesel unsaturation, which directly influences oxidative stability [42]. Biodiesel with higher iodine values generally displays lower oxidative stability.

Saponification value is another important characteristic of biodiesel quality. Asadi et al. (2020) reported saponification values of 49.5 and 71.68 for *Chlorella sorokiniana* and 91.5 and 93.64 for *Chlorella vulgaris* during the first and second cultivation stages, respectively, using non-sterile dairy wastewater as the growth medium [43]. In the present study, the saponification value ranged from 201 to 205 mg KOH/g, and was not significantly affected by the imposed stress conditions.

Effect of Stress on Biodiesel Quality Parameters

The cetane number, which reflects combustion quality, has been widely evaluated in biodiesel research. The cetane number represents a differential index characterized by the ignition delay and combustion performance. Global standards recommend minimum cetane numbers between 47 and 51 for acceptable engine performance.

Degree of unsaturation is another parameter affecting oxidative stability and long-term storage. Oils with lower unsaturation levels are more stable during prolonged storage. In our experiment, the lowest DU values were recorded in the control treatment at 250 bar/50 °C and 300 bar/70 °C.

Generally, ideal biodiesel quality is associated with longer-chain fatty acids and higher cetane numbers [44-45]. Higher proportions of LCSF typically enhance cetane value, resulting in shorter ignition delay and improved combustion efficiency [46].

Effect of Temperature and Pressure on Lipid Extraction

Temperature and pressure of supercritical CO_2 are known to influence extraction efficiency. Our findings revealed that increasing pressure enhanced extraction yield and reduced the time required to recover lipids, whereas increasing temperature showed an inverse trend—yield increased for certain parameters but decreased for others. Similar observations were reported by Goyeneche et al. (2018) during supercritical CO_2 extraction from *Raphanus sativus*, where higher pressure significantly improved extraction kinetics due to increased CO_2 density and solubility [47]. Ghasemi et al. (2011) likewise documented enhanced extraction yields from *Myrtus communis* leaves at higher pressures [48].

Zermane et al. (2014) also demonstrated that increased pressure maximized oil yield under all experimental conditions, while elevated temperature at constant pressure tended to reduce extraction efficiency due to alterations in solubility and fluid fugacity [49].

Conclusion

The results of this study demonstrate that the highest lipid and fatty acid yields from Chlorella vulgaris were obtained through a two-stage cultivation strategy incorporating nutrient deprivation, alkaline stress (pH 11), and salinity stress (2 M NaCl) for 24 hours. The optimized extraction conditions—supercritical CO_2 at 350 bar and 50 °C—provided maximum lipid recovery and fatty acid conversion efficiency.

Moreover, the produced biodiesel met all major quality parameters within the ASTM (USA) and EN (European Union) specifications, confirming its suitability for practical application. Overall, the combination of stress-induced lipid enhancement with supercritical CO_2 extraction represents an efficient and economically feasible approach for producing high-grade biodiesel from microalgal biomass, offering a sustainable route toward renewable energy development.

Conflict of Interest

The authors declare no conflict of interest.

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