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Research Article

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Fatty acid profile analysis of *Chlorella vulgaris* under salinity and cadmium stress: Feedstock characterization for biodiesel suitability

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ABSTRACT

Cultivation of microalgae *Chlorella vulgaris* under salinity and heavy metal stress can affect the type and quantity of fatty acids in its cells. This study aims to analyze the fatty acid profile of microalgae *C. vulgaris* cultivated in media containing 0.6 M NaCl and 95 µM CdCl2 based on the suitability of biodiesel raw material standards. The study began with determining the starter time of microalgae *C. vulgaris* culture, cultivation in salinity and cadmium stress medium, determining the growth phase of treatment and control, biomass extraction for metabolomics analysis, UPLC-MS/MS, fatty acid classification using LIPID MAPS Structure Database (LMSD), and analysis of biodiesel quality standards based on ASTM D6751 and EN14214 which include cetane number (CN), saponification value (SV), cold filter plugging point (CFFP), degree of unsaturation (DU), iodine value (IV) and long-chain saturated factor (LCSF). The results showed an increase in the degree of monounsaturated fatty acids (MUFA) by 10.7 % and a decrease in polyunsaturated fatty acids (PUFA) by 36.71% which played a role in the stability of biodiesel compared to the control treatment. The results of the analysis of CN, IV, and CFPP were respectively 55.4, 59, and -16.5. This shows that the composition of fatty acids formed in the microalgae extract *C. vulgaris* with salinity and cadmium stress treatment (0.6 M NaCl, 95 µM CdCl2) has the potential to be a source of quality biodiesel raw materials.

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INTRODUCTION

The world's energy demand continues to increase in line with population growth and industrial development (Zhang et al., 2022; Patnaik & Mallick, 2021). Biodiesel is one of the alternative solutions that are environmentally friendly and sustainable (Rafa et al., 2021). One of the sources of biomass that has great potential in the production of biodiesel is microalgae (Mallick et al., 2016). Biodiesel derived from microalgae has several advantages compared to terrestrial plant-based biodiesel, such as fast growth, efficient in absorbing carbon dioxide, and can grow in various environmental conditions without competing with agricultural land (Mata et al., 2009; Brennan & Owende, 2009). However, the quality of biodiesel is highly dependent on the composition of the fatty acids possessed by these microalgae (Hawrot-Paw et al., 2021). Saturated fatty acids (SFAs) provide good oxidative stability, but can increase the freezing point of biodiesel. On the other hand, unsaturated fatty acids (MUFAs and PUFAs) contribute to the nature of fuel flow at low temperatures, but are more susceptible to oxidation (Hawrot-Paw et al., 2021; Wahlen et al., 2012).

Microalgae have a high lipid content and the ability to grow in a variety of environmental conditions (Mohammadi & Azizollahi-Aliabadi, 2013). Although microalgae are known to be able to produce high amounts of lipids, mass lipid productivity at the industrial level is still not optimal because natural strains do not always produce high levels of lipids and optimal cultivation conditions for growth are often different from optimal conditions for lipid accumulation where lipids are usually produced under stress conditions (Park et al. 2019). Research to develop superior strains that are stable, stress-resistant, and able to consistently produce large amounts of lipids, as well as cultivation strategies that do not sacrifice biomass growth, needs to be carried out.

One of the most researched microalgae species is *Chlorella vulgaris*. This species has a diverse content of fatty acids and can be modified by various environmental factors (Garcia, 2012). Environmental stresses such as salinity and heavy metals can affect their fatty acid composition. This composition can also affect the quality of the biodiesel produced (Al-Lwayzy et al., 2014). Heavy metals such as cadmium (Cd) are known to cause oxidative stress in microalgae. This stress impacts cellular metabolic changes such as lipid biosynthesis (Ranjbar & Malcata, 2022). In addition, salinity also plays a role in influencing fatty acid composition through an adaptation mechanism involving a change in the balance between saturated fatty acids/SFA, monounsaturated fatty acids/MUFA, and polyunsaturated fatty acids/PUFAs (Song et al., 2024). This change is important in assessing the quality of biodiesel through the ratio of SFA, MUFA, and PUFA produced.

This ratio affects the physicochemical properties of the fuel, such as oxidative stability, viscosity, cetane number, and cold flow properties (Song et al., 2024; Shi et al., 2020). Several metabolic engineering studies using abiotic stress treatments to change the fatty acid composition of microalgae to become potential biodiesel raw materials have been conducted. However, research with a combination of salinity treatment with cadmium heavy metal on *C. vulgaris* for the purpose of evaluating the fatty acid composition as a potential biodiesel raw material has never been conducted. This research will also open up the potential for biorefinery and heavy metal bioremediation by *C. vulgaris*. This study aims to analyze the fatty acid profile of microalgae *C. vulgaris* cultivated in a medium containing 0.6 M NaCl and 95 µM $CdCl_2$ based on the suitability of biodiesel standards. By understanding the composition of fatty acids that change due to stress, this study is expected to provide insight into the optimization of microalgae-based biodiesel production under sub-ideal environmental conditions.

RESEARCH METHODS

This study is an experimental study that analyzes whether the composition of fatty acids produced by *C. vulgaris* microalgae cells that have been treated in a medium containing 0.6 M NaCl and 95 µM CdCl2 has the potential as a raw material for biodiesel based on the characteristics of cetane number (cn), saponification value (sv), iodine value (iv), degree of unsaturation (du), long-chain saturated factor (lcsf) and cold filter plugging point (cfpp). The instruments and reagents needed in this research include low speed cryogenic universal centrifuge (centrifuge 5430， eppendorf), vortex mixer (ql-901, kylin-bell lab instruments co., ltd china), ultra pure water meter (milli-q integral, millipore corporation, usa), refrigerated vacuum concentrator (maxi vacbeta, gene company), tissue grinder (jxfstprp, shanghai xin ning， china), methanol (a454-4), acetonitrile （a998-4） were all LCMS level (thermo fisher scientific, usa), ammonia formate (17843-250g， honeywell fluka, usa), formic acid （50144-50 ml， dimka， usa), the water was supplied by a ultra pure water meter. internal standard : d3-leucine,13c9-phenylalanine,d5-tryptophan,13c3-progesterone.

Microalgae cultivation is carried out using the following methods, a total of 20% (v/v) of 6-day-old *C. vulgaris* microalgae starter culture was cultivated in the control treatment in vitro in artificial seawater media Blue Treasure™, added with sterile Walne nutrients as much as 1 ml/liter. Cultivation conditions were carried out at a temperature of 25˚C, medium pH 7.5-8, continuous irradiation for 24 hours, bubbled oxygen with an aerator, CO_2 levels of 2%, light intensity of 4000 lux, and salinity of 35 ‰. Cultivation in the treatment media was carried out by adding 0.6 M NaCl and 95µM CdCl2. Microalgae growth in the control and stress treatments was carried out by calculating cell density using a Neubauer's chamber Haemocytometer, weighing dry biomass, and measuring optical density (OD) with a UV-Vis spectrophotometer at λ 680 nm, every 24 hours until reaching the death phase (Gonzalez-Esquer et al., 2019).

Microalgae harvesting was carried out at the logarithmic phase by separating the microalgae from the medium using a centrifuge at 3000 rpm for 10 minutes. A total of 1-10 mg of filtered biomass was then diluted with 25 mL of 0.5 M ammonium formate to remove salt residues in the cell culture. Furthermore, filtration was carried out, and this step was carried out twice (Barten et al., 2022). The collected microalgae biomass was then dried using a freeze dryer until it became a dry powder. Metabolite extraction for metabolomic analysis was performed as follows, 50 µg of samples were weighed into 1.5 mL Eppendorf tubes respectively and soaked with 800 µL of precooling extraction solution (methanol: H2O = 7:3, v/v) and 20 µL Internal Standard 1 (IS1). Homogenization was conducted with a weaving grinder at 50 Hz for 10 minutes and then water bath ultrasonication at 4 °C for 30 minutes. Following the standing still at – 20 °C for 1 hour, the extracts were centrifuged at a speed of 14000 rpm at 4 °C for 15 minutes. 600 µL of the supernatant was filtered with a 0.22 µm membrane, and 20 µL of filtered solution from each sample was composited into the mixed QC sample to evaluate the repeatability and stability of LC/MS analysis. Filtered samples and mixed QC samples were transferred to the 1.5 mL sample vials for instrument running.

This experiment used waters UPLC I-Class Plus Waters, USA） tandom Q Exactive high resolution mass spectrometer (Thermo Fisher Scientific, USA) for the separation and detection of metabolites. Chromatographic conditions: Chromatographic separation was performed on Hypersil GOLD aQ Dim column (1.9 µm 2.1*100 mm, Thermo Fisher Scientific, USA), with mobile phase A consisting of 0.1% formic acid in water and mobile phase B consisting of 0.1 formic acid in acetonitrile. The column temperature was maintained at 40 °C. The gradient conditions were as follows: 5% B over 0.0-2.0 min,

5-95% B over 2.0-22.0 min, held constant at 95% B over 22.0-27.0 min, and washed with 95% B over 27.1-30 min. The flow rate was 0.3 mL/min, and the injection volume was 5 µL. Mass spectrometry conditions: using Q exactive (thermo fisher scientific, USA), perform primary and secondary mass spectrometry data acquisition. The scan range was 125~1500 m/z for positive ions and 100–1500 m/z for negative ions with a resolution of 70000, and the automatic gain control (AGC) target for MS acquisitions was set to 1e6 with a maximum ion injection time of 100 ms. The top 3 precursors were selected for subsequent MSMS fragmentation with a maximum ion injection time of 50 ms and resolution of 30000; the AGC was 2e5. The stepped normalized collision energy was set to 20, 40, and 60 eV. ESI parameters were set as: Sheath gas flow rate was 40, Aux gas flow rate was 10,positive-ion mode Spray voltage(|KV|) was 3.80, negative-ion mode Spray voltage(|KV|) was 3.20, Capillary temperature was 320°C, Aux gas heater temperature was 350°C.

After importing the off-line data of mass spectrometry into compound discoverer 3.3 (Thermo Fisher Scientific, USA) software and analyzing the mass spectrometry data in combination with bmdb (BGI metabolome database), mzcloud database and chemspider online database, a data matrix containing information such as metabolite peak area and identification results will be obtained. After that, the table will be further analyzed and processed. Software information: compound discoverer version v.3.3 parameter : parent ion mass deviation: < 5ppm mass deviation of fragment ions: <10ppm retention time deviation: < 0.2min official website : https://mycompounddiscoverer.com/. Fatty acid classification was performed using the LIPID MAPS Structure Database (LMSD) (https://www.lipidmaps.org/databases/lmsd/browse) to filter relevant compounds from identified metabolites in R (version 4.4.2). Fatty acids were categorized into unsaturated, straight-chain, and branched-chain types, with saturated fatty acids identified from both the straight-chain and branched-chain categories. LC-MS results were then systematically classified into three main groups: saturated, unsaturated, and conjugated fatty acids. The classification was based on exact molecular formula matching and structural similarity assessments. The key R package used in this process was dplyr.

Analysis of biodiesel quality is carried out by calculating parameters including the cetane number (CN), saponification value (SV), iodine value (IV), degree of unsaturation (DU), long-chain saturation factor (LCSF), and cold filter plugging point (CFPP). All parameter values were calculated based on empirical equations in the study of Francisco et al. (2010). The results are compared with the international biodiesel standards ASTM D6751 and EN 14.214. Cetane number, saponification value, and iodine value are calculated according to equations 1–3.

$$CN = 46{,}3 + \frac{5458}{SV} - (0{,}225 \times IV), \quad (1)$$

$$SV = \frac{\Sigma(560 \times N)}{M} \quad (2)$$

$$IV = \frac{\Sigma(254 \times D \times N)}{M} \quad (3)$$

Where CN is the cetane number, SV is the saponification value, IV is the iodine value, *D* is the number of double bonds, *M* is the molecular mass, and *N* is the percentage of each fatty acid component. The degree of unsaturation is calculated from empirical equation 4 by considering the amount of monounsaturated and polyunsaturated methyl esters (wt%) present in microalgae oil.

$$DU = MUFA + (2 \times PUFA) \quad (4)$$

Where DU is the unsaturation degree (%), MUFA is the weight percentage of monounsaturated fatty acids (wt%), and PUFA is the weight percentage of polyunsaturated fatty acids (wt%). The long-chain saturated factor is obtained from the empirical equation 5 by considering fatty acids and giving more

weight to the composition of fatty acids with long chains. This parameter correlates with the cold filter plugging point, using equation 6.

$$LCFF = (0,1 \times C16)+(0,5 \times C18)+(1 \times C20)+(1,5 \times C22)+(2 \times C24) \quad (5)$$

$$CFPP = (3,1417 \times LCSF) – (16,477) \quad (6)$$

Where LCSF is a long-chain saturated factor; C16, C18, C20, C22, and C24 are the weight percentage of each fatty acid (wt%), and CFPP is the cold filter plugging point.

FINDING AND DISCUSSION

The growth performance of *Chlorella vulgaris* under combined cadmium (95 µM $CdCl_2$) and salinity (0.6 M NaCl) stress compared to control conditions is presented in Figure 1. The cell density was monitored daily for 15 days, with three replicates for each treatment to ensure statistical reliability. The stress treatment (A) consistently showed lower cell density compared to the control (E) throughout the cultivation period, demonstrating the inhibitory effects of combined environmental stressors. The average growth rate for the stress treatment resulted in a final cell density of 0.48/day, which is a 40.29% reduction compared to the control's final cell density of 0.8/day under non-stressed conditions.

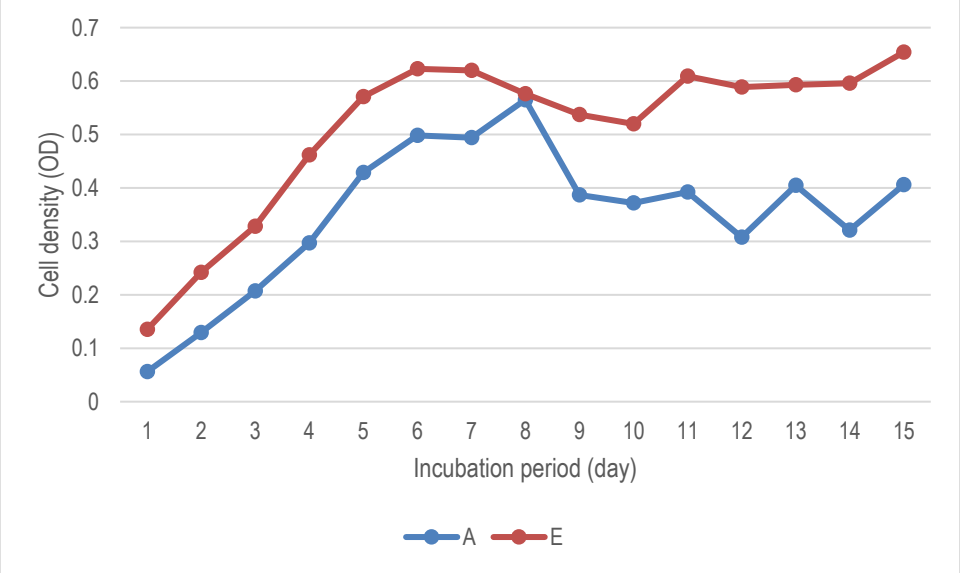

Figure 1. Growth Curves of *Chlorella vulgaris* under Cadmium and Salinity Stress (A) and Control (E) Conditions Over 15 Days of Cultivation

The initial stimulation followed by growth inhibition observed in *C. vulgaris* under combined Cd and salinity stress aligns with findings from previous studies on microalgae stress responses. Algburi et al. (2024) reported similar biphasic growth patterns in *C. vulgaris* exposed to heavy metal stress, where low concentrations initially stimulate growth through hormesis effects before toxicity becomes apparent. The tolerance mechanism of *C. vulgaris* involves multiple physiological adaptations, including the activation of antioxidant systems and metal sequestration processes (Carfagna et al., 2013). These mechanisms include the synthesis of metallothioneins, phytochelatins, and enhanced production of enzymatic antioxidants such as superoxide dismutase and catalase. The energy cost associated with these protective mechanisms may explain the eventual decline in cell density observed in our study. Furthermore, cellular resources diverted to stress response pathways reduce the energy available for

normal growth and reproduction processes, ultimately limiting biomass accumulation under prolonged stress exposure.

The growth inhibition observed in later stages is consistent with the synergistic toxic effects of combined stressors documented in microalgae literature. Cadmium toxicity primarily affects photosynthetic processes by disrupting chlorophyll synthesis and electron transport chains, leading to reduced photosynthetic efficiency (Shim et al., 2009; Rosyadi et al., 2022). Simultaneously, high salinity stress causes osmotic imbalance and ion toxicity, particularly affecting K^+/Na^+ homeostasis in algal cells and disrupting membrane integrity (Ding et al., 2013). The combination of these stressors can overwhelm cellular defense mechanisms, resulting in reduced growth rates as observed in our study. This synergistic effect has been well-documented in stress physiology research, where multiple stressors often produce effects greater than the sum of individual stressor impacts. The cellular energy budget becomes critically strained when multiple stress response pathways are activated simultaneously, leaving insufficient resources for normal metabolic processes and growth maintenance.

Ouyang et al. (2012) demonstrated that *Chlorella* species can tolerate moderate Cd concentrations through enhanced synthesis of metallothioneins and phytochelatins, which bind and sequester heavy metals in cellular compartments. However, when combined with salinity stress, the energy allocation for multiple stress responses may compromise overall growth performance and metabolic efficiency. This finding corroborates the results of Rachlin & Grosso (1991), who reported that combined environmental stresses often produce more severe effects than individual stressors. The metabolic burden of maintaining multiple stress response systems simultaneously creates a significant drain on cellular ATP reserves and biosynthetic capacity. Additionally, the interference between different stress response pathways can reduce the effectiveness of individual adaptation mechanisms. The concentration thresholds used in our study (95 µM $CdCl_2$ and 0.6 M NaCl) appear to exceed the combined tolerance capacity of *C. vulgaris*, explaining the observed growth inhibition pattern.

The dry weight measurements and biomass productivity calculations were conducted on day 15 of cultivation, representing the cumulative effects of stress exposure over the entire growth period (Figure 2). Under stress conditions, the dry weight reached 0.001 g/L with a productivity of 0.000617 g/L/day, while the control achieved 0.001033 g/L with a productivity of 0.000243 g/L/day. Despite the lower absolute biomass, the stress treatment showed efficient resource utilization patterns characteristic of stress-adapted microalgae. The biomass reduction of 3,23% under stress conditions reflects the metabolic cost of maintaining cellular homeostasis under adverse environmental conditions. This reduction is within the range reported for other microalgae species under similar stress intensities, indicating that *C. vulgaris* maintains reasonable productivity even under challenging conditions. The maintained viability and continued biomass accumulation under stress demonstrate the inherent resilience of this species to environmental perturbations.

The reduced biomass under stress conditions reflects the energy cost of stress adaptation mechanisms that compete with growth-related processes for cellular resources. *C. vulgaris* allocates significant metabolic resources to synthesize protective compounds such as proline, glycine betaine, and antioxidant enzymes under salinity stress (Annamalai et al., 2016; Sari et al., 2024). Additionally, cadmium detoxification requires energy for the synthesis of metal-binding peptides and active transport of metals to vacuolar compartments (Nowicka, 2022; Wahyu et al., 2020). The synthesis of stress proteins, including heat shock proteins and chaperones, further contributes to the metabolic burden under adverse conditions. These protective mechanisms are essential for cell survival but come at the cost of reduced growth efficiency and biomass accumulation. The observation that cells continue to accumulate biomass

despite these challenges demonstrates the effectiveness of *C. vulgaris* adaptation strategies and its potential as a robust feedstock organism.

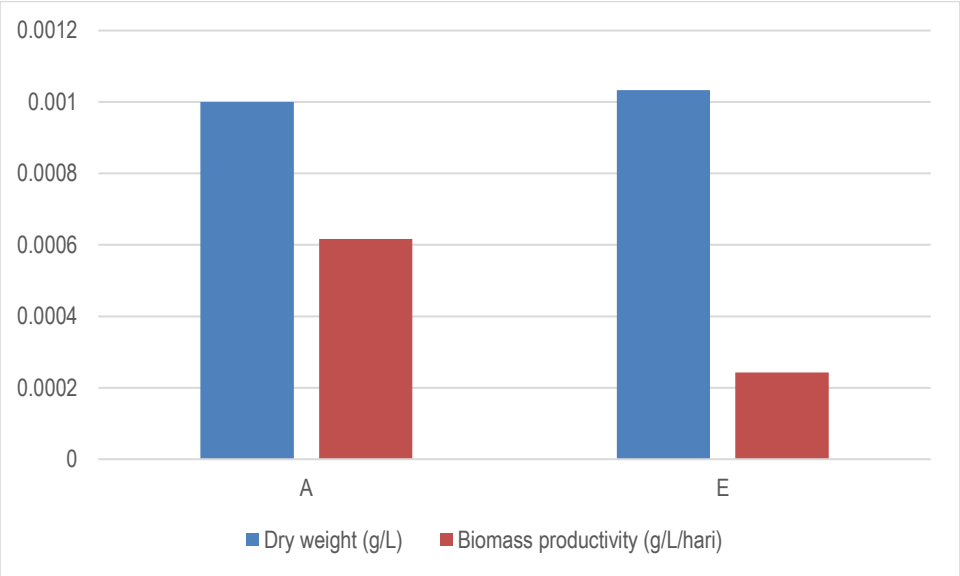

Figure 2. Biomass (Dry Weight) and Biomass Productivity of *Chlorella vulgaris* under Cd and Salinity Stress (A) and Control (E) Conditions

The biomass productivity patterns observed align with findings from Yun et al. (2019), who reported that moderate salinity stress (30 mM NaCl) initially stimulates *C. vulgaris* growth but higher concentrations lead to biomass reduction. The study demonstrated that stressed algae often exhibit altered metabolic profiles, including increased lipid accumulation as a stress response mechanism. This metabolic shift may explain the maintained productivity despite lower total biomass in our stress treatment, as cells redirect carbon flow toward storage compounds. The phenomenon of stress-induced lipid accumulation has been widely reported in microalgae and represents an important adaptation strategy for surviving unfavorable conditions (Pandit et al., 2017). Under nutrient limitation or environmental stress, many microalgae species shift from protein and carbohydrate synthesis toward lipid biosynthesis and accumulation. This metabolic reprogramming not only serves as a survival mechanism but also enhances the value of the biomass for biodiesel production applications.

Liquid Chromatography Mass Spectrometry (LCMS) or MS analysis identified 1,843 untargeted metabolites, from which fatty acid compounds were specifically extracted and classified using the LIPID MAPS Structure Database (LMSD). The comprehensive metabolomic approach ensured accurate identification and quantification of fatty acid species across different structural classes. The fatty acid profiles for both treatments are presented in Table 1, showing significant compositional changes under stress conditions that reflect adaptive cellular responses. This untargeted approach provides a more complete picture of fatty acid metabolism compared to traditional targeted analyses that focus on specific compounds. The large number of detected metabolites demonstrates the metabolic complexity of microalgae and the extensive biochemical changes that occur under environmental stress. The classification system used allows for systematic comparison of fatty acid profiles and provides insights into the metabolic pathways affected by stress conditions.

Table 1. Fatty Acid Profile of *Chlorella vulgaris* under Cd and Salinity Stress (A) and Control (E) Conditions with repetition.

Name	LCMS Formula	Peak Area						Double Bonds	Classification
		A1	A2	A3	E1	E2	E3		
(2S,4S)-Pinnatanine	C10 H16 N2 O5	10.413.480	11.749.552	25.113.769	2.610.201	4.173.926	4.071.920	2	Unsaturated
Cladosporacid E	C10 H16 O4	15.606.969	999.707	306.123	1.388.656	1.236.960	401.261	2	Unsaturated
2,6R-Dimethyl-2E-octen-1,8-dioic acid	C10 H16 O4	7.149.811	141.944	136.360	1.739.429	364.816	110.811	2	Unsaturated
10-fluoro-capric acid	C10 H19 F O2	47.327.875	37.154.733	82.400.919	77.561.711	37.816.925	20.482.891	0,5	Unsaturated
Pantheric Acid C	C11 H18 O4	22.182.913	5.663.361	5.171.098	37.032.098	11.082.925	2.640.560	2	Unsaturated
8-propionyl caprylic acid	C11 H20 O3	10.612.301	2.390.694	1.572.770	9.001.443	4.813.651	2.154.834	1	Unsaturated
Jasmonic acid	C12 H18 O3	21.104.566	306.795	151.418	738.559	1.560.225	539.527	3	Unsaturated
Mycinonic acid III	C13 H20 O4	17.863.025	129.807	434.455	5.575.503	1.561.390	450.429	3	Unsaturated
Propyl 2,4-decadienoate	C13 H22 O2	6.045.806	4.723.980	6.190.808	6.701.062	10.543.502	12.527.703	2	Unsaturated
5-oxo-6E,8Z-tetradecadienoic acid	C14 H22 O3	9.378.490	820.037	408.983	5.857.064	2.324.769	266.810	3	Unsaturated
13-tetradecynoic acid	C14 H24 O2	450.400	501.767	41.032.694	506.558	519.272	11.882.674	2	Unsaturated
7-oxo-11E-Tetradecenoic acid	C14 H24 O3	9.138.052	3.463.891	4.583.127	2.417.190	6.331.074	7.644.542	2	Unsaturated
14-hydroxy-12-tetradecenoic acid	C14 H26 O3	2.120.138	5.840.910	12.397.410	1.119.788	15.511.950	16.838.418	1	Conjugated/Modified
2-Hydroxymyristic acid	C14 H28 O3	812.412	1.258.913	10.819.195	1.260.122	2.343.274	35.181.299	0	Straight Chain Saturated
Ipurolic acid	C14 H28 O4	923.398	1.958.632	88.485.092	149.934	10.032.218	146.633.500	0	Straight Chain Saturated
Gallicynoic acid B	C15 H24 O4	10.704.131	138.693	89.617	3.817.867	1.044.695	406.327	3	Unsaturated
Octyl phenylacetate	C16 H24 O2	2.776.052	6.725.408	5.021.220	4.159.788	6.787.424	8.873.402	4	Unsaturated
Tetranor-12R-HETE	C16 H26 O3	11.487.987	26.817.439	30.097.649	4.075.305	29.462.034	26.946.419	3	Unsaturated
9-oxo-7E-hexadecenoic acid	C16 H28 O3	163.235.649	76.575.821	37.635.118	40.478.760	95.243.313	47.476.109	2	Unsaturated
Palmitic acid	C16 H32 O2	7.520.971	7.594.648	32.485.598	3.756.927	14.240.355	83.499.382	0	Straight Chain Saturated
Heptadecanedioic acid	C17 H32 O4	65.392.823	55.270.890	168.164.757	118.903.883	59.727.954	20.352.901	1	Unsaturated
1-(4-carboxybutanamido)-1'-	C18 H22 Fe N2 O4	9.814.817	8.491.171	5.974.625	608.240	830.704	983.383	7	Unsaturated

Name	LCMS Formula	Peak Area						Double Bonds	Classification
		A1	A2	A3	E1	E2	E3		
(dimethylcarbamoyl)ferrocene									
12-oxo-14,18-dihydroxy-9Z,13E,15Z-octadecatrienoic acid	C18 H28 O5	7.659.945	585.758	554.424	1.986.136	1.563.227	506.844	4	Conjugated/Modified
alpha-Linolenic acid	C18 H30 O2	758.129.071	367.024.791	58.707.832	702.414.740	625.524.183	475.009.525	3	Unsaturated
Linoleic acid	C18 H32 O2	13.960.695	45.290.727	7.583.440	12.696.521	16.024.523	33.403.245	2	Unsaturated
Linoelaidic acid	C18 H32 O2	175.088.358	145.100.969	62.328.456	234.712.023	310.594.463	360.732.360	2	Unsaturated
Linoleamide	C18 H33 N O	162.787.615	37.996.519	121.153.313	64.605.745	81.419.417	61.280.536		Unknown
Oleic acid	C18 H34 O2	11.249.533	6.646.052	2.791.296	12.446.517	47.952.609	52.981.807	1	Unsaturated
Floionic acid	C18 H34 O6	15.884.338	23.170.384	3.061.678	13.675.953	10.452.893	6.996.660	1	Unsaturated
Oleamide	C18 H35 N O	11.798.507.560	4.440.385.425	8.594.172.124	6.517.146.334	7.178.245.274	8.575.754.103		Unknown
methyl 9-hydroperoxy-10,12-epidioxy-13,15-octadecadienoate	C19 H32 O6	205.191	666.619	43.055.946	163.751	1.417.504	22.577.856	3	Unsaturated
3-oxo-nonadecanoic acid	C19 H36 O3	2.306.139.507	4.253.252.491	2.666.697.153	3.837.738.142	1.509.192.397	4.486.454.048	1	Unsaturated
12,13-dihydroxy-11-methoxy-9-octadecenoic acid	C19 H36 O5	25.665.120	35.387.032	26.531.643	8.451.719	13.834.615	25.807.019	1	Conjugated/Modified
N-palmitoyl serine	C19 H37 N O4	4.043.459	21.689.004	16.237.283	83.204.390	1.603.584	7.372.764	0,5	Unsaturated
Cissoic acid	C20 H30 O10	6.100.313	1.791.137	1.153.963	11.688.374	3.716.815	747.240	5	Unsaturated
15-OxoEDE	C20 H34 O3	5.281.791	39.021.036	18.655.754	215.895.941	77.567.737	7.337.036	3	Unsaturated
N-(9Z-hexadecenoyl)-homoserine lactone	C20 H35 N O3	3.189.078	646.361	308.347	11.399.151	1.348.998	1.517.038	2,5	Unsaturated
Ethyl oleate	C20 H38 O2	4.031.618	1.455.596	1.155.525	1.494.978	1.393.673	11.234.255	1	Unsaturated
N-oleoyl alanine	C21 H39 N O3	21.131.858	10.354.420	5.387.688	11.939.642	4.961.272	14.243.695	1,5	Unsaturated
Ascorbyl palmitate	C22 H38 O7	6.468.861	3.422.818	3.921.984	2.303.034	5.650.453	5.206.992	3	Unsaturated
N-isobutyl-2E,4Z-octadecadienoyl amine	C22 H41 N O	16.531.341	19.782.323	31.829.069	30.814.526	14.845.226	155.442.054		Unknown

Name	LCMS Formula	Peak Area						Double Bonds	Classification
		A1	A2	A3	E1	E2	E3		
N-(3-hydroxy-octadecanoyl)-homoserine lactone	C22 H41 N O4	899.007	2.022.046	5.164.823	1.162.080	5.483.857	1.289.074	1,5	Conjugated/Modified
Erucamide	C22 H43 N O	398.257.462	373.390.565	799.099.068	599.563.766	336.985.925	3.903.446.740		Unknown
N-(9,12-octadecadienoyl)-glutamic acid	C23 H39 N O5	1.158.388	15.730.943	17.965.384	2.271.049	1.324.325	872.997	3,5	Unsaturated
N,N-(2,2-dihydroxy-ethyl) arachidonoyl amine	C24 H41 N O3	10.523.742	2.114.514	171.814	14.976.943	19.218.838	18.013.293	3,5	Conjugated/Modified
Ferroxamine	C25 H50 Fe N6 O8	242.466	4.220.543	51.683.546	344.796	1.233.462	1.597.768	0	Straight Chain Saturated
ELV-N34	C34 H56 O4	637.093	1.576.881	160.962	374.962	3.099.101	8.284.825	6	Unsaturated
Succinic acid	C4 H6 O4	1.145.488.971	2.180.777.296	6.606.601.200	1.436.923.058	1.136.710.050	1.544.674.374	1	Unsaturated
Nepheliosyne A	C47 H70 O11	3.626.943	3.883.363	4.515.220	27.866.905	3.136.714	2.958.159	12	Unsaturated
Nepheliosyne B	C47 H70 O11	159.053	179.394	260.865	141.226	5.703.467	2.035.921	12	Unsaturated
Osirisyne H	C47 H72 O10	17.096.010	1.605.304	6.540.393	17.877.855	8.925.471	12.648.255	11	Unsaturated
Fulvyne F	C47 H72 O11	22.811.647	6.400.540	645.074	48.331.581	14.406.684	14.037.797	11	Unsaturated
Glutaconic acid	C5 H6 O4	2.941.888	8.143.158	33.748.512	2.776.733	7.251.235	8.774.354	2	Unsaturated
Adipic acid	C6 H10 O4	1.499.546	1.466.141	499.341	569.646	22.164.338	8.977.194	1	Unsaturated
Mevalonic acid	C6 H12 O4	25.854.323	21.022.267	79.855.409	49.372.083	31.165.479	38.141.073	0	Straight Chain Saturated
Pimelic acid	C7 H12 O4	12.165.855	6.971.365	34.541.559	5.987.932	9.312.040	6.000.073	1	Unsaturated
Suberic acid	C8 H14 O4	4.279.095	325.486	418.191	555.605	1.283.607	463.356	1	Unsaturated
8-aminocaprylic acid	C8 H17 N O2	1.781.183	1.464.546	6.238.319	865.516	5.799.842	2.859.617	-0,5	Unknown
Azelaic acid	C9 H16 O4	147.049.936	135.779.027	139.276.730	97.807.195	100.492.683	107.985.753	1	Unsaturated
3R-aminononanoic acid	C9 H19 N O2	32.038.857	5.527.374	441.990	5.064.751	5.365.627	645.191	-0,5	Unknown
Hexadecanedioic acid	C16 H30 O4	10.391.097	26.509.629	5.380.976	5.499.598	31.504.094	19.536.121	1	Unsaturated
2S-hydroxy-3-(10Z-tetradecenoyloxy)-propanoic acid	C17 H30 O5	17.773.072	9.318.072	1.960.081	1.090.732	4.292.960	5.362.796	2	Conjugated/Modified
Tricosanoic acid	C23 H46 O2	27.728.428	64.080.469	230.454.405	69.832.604	17.711.536	75.480.896	0	Straight Chain Saturated

The fatty acid composition analysis revealed distinct patterns between stress and control treatments, with particularly notable changes in the monounsaturated and polyunsaturated fatty acid fractions (Table 2, Figure 3). Under stress conditions, monounsaturated fatty acids (MUFA) increased from 76.12% (control) to 84.26% (stress), representing a substantial 10.7% increase in relative abundance. Conversely, polyunsaturated fatty acids (PUFA) decreased from 21.05% (control) to 13.32% (stress), showing a dramatic 36.71% reduction that indicates significant metabolic reorganization. Saturated fatty acids (SFA) remained relatively stable with only minor variations between treatments, suggesting that these structural lipids are tightly regulated. The magnitude of these changes indicates a fundamental shift in fatty acid metabolism under stress conditions, reflecting both adaptive responses and metabolic constraints. These compositional changes have significant implications for the physicochemical properties of extracted lipids and their suitability for biodiesel production.

Table 2. Composition of Fatty Acids (%) of *Chlorella vulgaris* under Cd and Salinity Stress (A) and Control (E) Conditions with repetition

Fatty Acid	Stress (A) %				Control (E) %			
	A1	A2	A3	A	E1	E2	E3	E
SFA	1.23	1.32	4.69	2.61	1.77	1.80	4.92	2.67
MUFA	72.83	88.48	91.48	84.26	78.48	68.77	81.11	76.12
PUFA	25.94	10.20	3.83	13.32	19.76	29.43	13.96	21.05

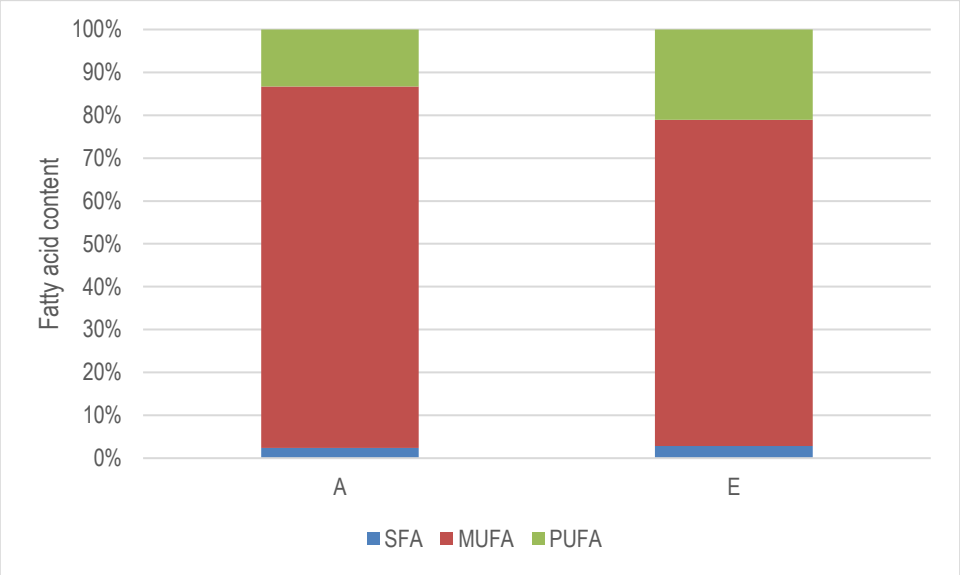

Figure 3. Composition of Fatty Acids (%) of *Chlorella vulgaris* under Cd and Salinity Stress (A) and Control (E) Conditions

The observed changes in fatty acid composition represent adaptive responses to environmental stress that have been documented across various microalgae species. The increase in MUFA content under stress conditions aligns with membrane adaptation strategies reported in several microalgae species, where cells modify lipid composition to maintain membrane functionality. Menon et al. (2013) demonstrated that *Chlorella* species modify membrane fatty acid composition to maintain membrane fluidity and functionality under stress conditions. The increase in MUFA helps maintain optimal membrane properties while providing better oxidative stability compared to PUFA, which are more susceptible to peroxidation. This adaptation strategy represents a balance between maintaining membrane fluidity

necessary for cellular processes and protecting against oxidative damage. The preferential synthesis of monounsaturated over polyunsaturated fatty acids under stress conditions reflects the cellular priority of membrane stability over membrane fluidity optimization.

The reduction in PUFA content under combined Cd and salinity stress is consistent with findings from Chia et al. (2013), who reported decreased PUFA levels in *Chlorella* exposed to heavy metal stress. This reduction likely results from enhanced lipid peroxidation caused by reactive oxygen species (ROS) generated under stress conditions, leading to the degradation of existing PUFA molecules (Yusuf et al., 2022). Cadmium is known to induce oxidative stress by disrupting antioxidant enzyme systems and promoting ROS formation, which specifically target polyunsaturated fatty acids due to their multiple double bonds (Farag et al., 2023; Zsiros et al., 2020). The cellular response to this oxidative challenge involves reducing PUFA synthesis to minimize the substrate availability for lipid peroxidation reactions. Additionally, the energy cost of maintaining highly unsaturated fatty acids under oxidative stress conditions may favor the synthesis of more stable monounsaturated alternatives. This metabolic shift represents a protective mechanism that sacrifices membrane fluidity optimization in favor of oxidative stability and cellular survival.

The relatively stable SFA content suggests that *C. vulgaris* maintains essential membrane structural components while adjusting the unsaturated fatty acid profile to cope with environmental challenges. This pattern differs from some studies on halophilic microalgae where SFA content increases significantly under salinity stress (El-Sheekh et al., 2024). The species-specific response observed in our study indicates that *C. vulgaris* employs a different membrane adaptation strategy, prioritizing MUFA over SFA for stress tolerance. This adaptation strategy may reflect the evolutionary history and ecological niche of *C. vulgaris*, which has developed efficient mechanisms for maintaining membrane function without relying heavily on saturated fatty acid accumulation. The maintenance of SFA levels suggests that the basic membrane architecture remains intact while fine-tuning occurs in the unsaturated fatty acid composition. This selective modification approach allows for rapid metabolic adjustments without requiring extensive restructuring of membrane systems.

The biodiesel characteristics were evaluated based on fatty acid composition using established calculation methods for key parameters, including cetane number (CN), iodine value (IV), and cold filter plugging point (CFPP) according to ASTM D6751 and EN 14214 standards (Table 3). These parameters provide crucial information about fuel performance, including ignition quality, oxidative stability, and low-temperature operability. The calculations were performed using validated correlations that relate fatty acid composition to biodiesel properties, allowing for accurate prediction of fuel quality without requiring actual biodiesel synthesis. This predictive approach is widely used in biodiesel research and provides reliable estimates of fuel properties based on feedstock composition. The comprehensive analysis includes both mandatory parameters specified in international standards and additional quality indicators that affect fuel performance, covering all critical aspects of biodiesel quality that determine commercial viability and engine compatibility.

The results indicate that the control treatment (E) produced biodiesel with generally superior quality characteristics compared to the stress treatment (A). The cetane number (CN) for the control was 57.8, which is higher than the stress treatment's 55.4. Both values comfortably exceed the minimum requirements of ≥ 47 for ASTM D6751 and ≥ 51 for EN 14214, indicating excellent ignition quality. The higher CN in the control aligns with a potentially higher proportion of saturated fatty acids, which are known to contribute to better combustion properties (Bamgboye & Hansen, 2008).

Table 3. Predicted Biodiesel Characteristics of *Chlorella vulgaris* under Cd and Salinity Stress (A) and Control (E) Conditions with repetition. CN: Cetane Number, SV: Saponification Value, IV: Iodine Value, DU: Degree of Unsaturation, LCSF: Long-chain Saturated Factor, CFPP: Cold Filter Plugging Point.

Predicted biodiesel characteristics	Stress (A) %				Control (E) %				International Standards of Biodiesel	
	A1	A2	A3	A	E1	E2	E3	E	ASTM D6751#	EN 14,214*
CN	61.2	52.2	52.7	55.4	54.3	57.7	61.5	57.8	≥ 47	≥ 51
SV	215.1	240.3	286.4	247.3	220	223.5	209.8	217.8	-	-
IV (g I2 100 g-1)	46.4	74.6	56.1	59	74.6	57.9	48	60.2	-	≤ 120
DU (wt %)	36.2	65.4	51.5	51	57.5	45.4	41	48	-	-
LCSF (wt %)	0.004	0.006	0.016	0.01	0.003	0.012	0.041	0.02	-	-
CFPP (°C)	-16.5	-16.5	-16.4	-16.5	-16.5	-16.4	-16.3	-16.4	-13 to -5	≤ 5 / ≤ -20

Regarding oxidative stability, the iodine value (IV) for the control was 60.2 g I_2/100g, slightly higher than the stress treatment's 59 g I_2/100g. Both values are well below the EN 14214 standard of ≤ 120 g I_2/100g, indicating good oxidative stability. While a lower IV generally suggests better oxidative stability, the difference between the two treatments is marginal, implying both biodiesels possess acceptable storage stability. This is further supported by the degree of unsaturation (DU), which was 48% for the control and 51% for the stress treatment. A higher DU typically correlates with increased susceptibility to oxidation, so the slightly lower DU in the control group might suggest a marginally better oxidative stability in that sample (Lin & Wu, 2022). The cold filter plugging point (CFPP) remained remarkably consistent for both treatments, at -16.5°C for the stressed sample and -16.4°C for the control. Both values are significantly lower than the EN 14214 standard of ≤ 5°C. This suggests that the biodiesel produced under these conditions would be suitable for use in a wide range of climatic conditions, including colder environments.

In summary, while both treatments yield biodiesel that meets international quality standards, the control condition (E) generally resulted in a biodiesel with a higher cetane number and marginally improved oxidative stability compared to the stress condition (A). This suggests that under the specific stress conditions applied, C. vulgaris may not enhance the overall biodiesel quality in terms of these key parameters, potentially due to shifts in fatty acid profiles that favor different characteristics. These findings are important for optimizing microalgal cultivation strategies for biodiesel production. The results of this study show both similarities and differences compared to previous research on microalgae biodiesel production under stress conditions. The improved cetane number and reduced iodine value observed in our stress treatment align with findings from El-Sheekh et al. (2024), who reported similar improvements in *Monoraphidium braunii* under salinity stress. However, their study showed increased SFA content, while our results demonstrate MUFA accumulation as the primary adaptation mechanism.

The biodiesel quality parameters obtained in this study compare favorably with commercial standards and previous microalgae studies. Lamaisri et al. (2015) emphasized that optimal biodiesel should contain balanced proportions of SFA, MUFA, and PUFA, with MUFA being the most desirable component. Our stress treatment achieved 84.26% MUFA content, which is exceptionally high compared to typical microalgae biodiesel feedstocks. The combined stress approach used in this study provides a more realistic simulation of environmental conditions compared to single-stress studies. Most previous research focused on individual stressors, but natural environments often present multiple simultaneous

challenges. Our results suggest that combined Cd and salinity stress creates synergistic effects that enhance biodiesel quality characteristics beyond what might be achieved with individual stressors.

The findings of this study have significant implications for sustainable biodiesel production using microalgae. The improved biodiesel quality under stress conditions suggests that controlled stress cultivation could be employed as a strategy to enhance feedstock quality. The higher cetane number, lower iodine value, and improved oxidative stability characteristics make the stressed *C. vulgaris* biomass particularly suitable for biodiesel production. The trade-off between growth rate and quality presents both challenges and opportunities for commercial applications. While stress conditions reduced the growth rate by 40.29%, the significant improvements in biodiesel quality parameters may justify this rate reduction in applications where fuel quality is prioritized. This approach could be particularly valuable for producing premium biodiesel grades or for applications requiring high oxidative stability. Furthermore, the ability of *C. vulgaris* to grow under combined heavy metal and salinity stress opens possibilities for utilizing marginal water sources or even wastewater for biodiesel production, contributing to both environmental remediation and renewable energy generation.

CONCLUSION

This study showed that the combination of cadmium and salinity stress affected the growth, fatty acid composition, and biodiesel quality of *Chlorella vulgaris*. The results showed an increase in the degree of monounsaturated fatty acids (MUFA) by 6.8 % and a decrease in polyunsaturated fatty acids (PUFA) by 37% which played a role in the stability of biodiesel compared to the control treatment. The results of the analysis of CN, IV, and CFPP were respectively 55.4, 59, and -16.5. This shows that the composition of fatty acids formed in the microalgae extract *C. vulgaris* with salinity and cadmium stress treatment (0.6 M NaCl, 95 µM CdCl2) has the potential to be a source of quality biodiesel raw materials.

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