Optimization of chlorophyll extraction from microalgae: evaluating solvent types, temperature, and time for biotechnological applications

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Abstract

Chlorophyll extraction plays a pivotal role in enhancing the functional properties of microalgal biomass for biofuel applications. This study investigated the efficiency of four commonly used solvents - methanol, ethanol, acetone, and diethyl ether in extracting chlorophyll from *Chlorella vulgaris* and *Scenedesmus quadricauda* under different experimental conditions. Extractions were conducted at three temperatures (20 °C, 40 °C, and 60 °C) and two-time intervals (5 and 10 minutes). Extraction efficiency was evaluated using the Extraction Efficiency Index, Temperature Sensitivity Index, Time Efficiency Index, Coefficient of Variation, and Comprehensive Extraction Index. Statistical analyses, including ANOVA and post-hoc tests, were conducted to identify significant differences at $p \leq 0.05$ among solvents and microalgae species. Methanol proved to be the most effective solvent for extracting chlorophyll. The highest chlorophyll concentrations were observed at 60 °C after 10 minutes, reached 21mg/L for *C. vulgaris* and 19.4 mg/L for *S. quadricauda*. Statistical analysis revealed significant differences ($p<0.001$) in chlorophyll extraction among the solvents, with methanol recorded as the best solvent. The EEI for methanol was 79.68% for *C. vulgaris* and 79.23% for *S. quadricauda,* indicating the highest extraction performance. Temperature significantly influenced extraction efficiency, with the highest yield at 60 °C. TEI and TSI confirmed that methanol had the highest extraction performance. Post-hoc analysis confirmed significant differences between methanol and other solvents.

Keywords: chlorophyll extraction, temperature sensitivity, extraction efficiency index, environmental conditions, solvent efficiency.

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Introduction

Microalgae are photosynthetic microscopic unicellular organisms capable to convert solar energy to chemical energy. These microorganisms exist individually or in chains or groups (Xie et al., 2022). They can be grown with simple growing requirement and their biomass can be used to produce human dietary supplements, animal feed and other beneficial substances for their high levels of protein, vitamins, pigments, and essential amino acid composition, which are not synthesized by human body (Abrha et al., 2025; Makaranga and Jutur, 2023; Wang et al., 2024b). *Chlorella* is a single celled green alga found in bodies of fresh water and contains high concentrations of nutrients such as vitamin C,

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minerals, carotenoids, vitamin B complex and ion. The algae also contain a high amount of protein and can produce healthy oils high in polyunsaturated fats used as a health supplement for a wide range of conditions. The algae also have the potential to treat bacteria, virus, and other conditions such as diabetes, cancer, and arthritis. Some cultures also believe that the algae can reverse the aging process if consumed in large enough quantities and cleanse the body (Naik et al., 2024).

Chlorophyll is one of the useful bioactive compounds that can be extracted from biomass of microalgae. It has been used as a natural food coloring agent and has antioxidant property (Zhou et al., 2022). Chlorophyll is a photosynthetic pigment present in green plants that absorb light energy and uses it to produce carbohydrates from carbon dioxide and water (Satpati and Pal, 2020). Chlorophyll is crucial to the process of photosynthesis, which is responsible for sustaining the light process of green plants. The skeleton of chlorophyll molecule is the porphyrin macrocycle, which comprises of four pyrrole rings (Mironov, 2019). In chlorophyll b, the methyl group in ring II of chlorophyll a is replaced by a formyl group (Sawicki et al., 2019).

There are multiple types of chlorophyll in plants. There are two main types of chlorophyll, chlorophyll a and chlorophyll b. Chlorophyll b and c are present in plants but are not involved in photosynthesis. Chlorophyll is a natural food coloring agent and is more expensive than artificial colorings (Ebrahimi et al., 2023). However, exposure of chlorophyll molecules to weak acids, oxygen, or light accelerates their oxidation and results in the formation of numerous degradation products (Qader and Shekha, 2023a; 2022; Qader and Shek, 2023; Qader and Shekha, 2023b).

This study compares the efficiency of ethanol, methanol, acetone, and diethyl ether for chlorophyll extraction from two widely studied microalgae: *Scenedesmus quadricauda* and *Chlorella vulgaris*. By optimizing the extraction process, this research aims to support the development of scalable and efficient chlorophyll extraction methods for biotechnological applications.

Materials and Methods

Microalgal cultivation

Algal samples **Chlorella vulgaris** and *Scenedesmus quadricauda* were inoculated on BG-11 medium containing 15% agar and were incubated at 25±2 °C, pH 8.2 and light intensity 3000-5000 lux 16hrs light and 8hrs dark for 14 days. The step was repeated many times to obtain purified algal species/ The purified algal colony was transferred to tubes contain 25 ml of BG-11 media and incubated under the same conditions mentioned above for 14 days to obtain algal inoculum. After the growth of algae, the culture was centrifuged with 3500 rpm for 10 min to separate the algae from the culture (Chen et al., 2011; Christenson and Sims, 2011; Qader and Shekha, 2023c; Qader and Shekha, 2023d). The supernatant was separated and sediment algae were put in sterilized dry petri dishes and left in room temperature to dry (Qader et al., 2025a; Sahu, 2014). Centrifugation is perhaps the most rapid and reliable method of recovering suspended algae (Olatunde et al., 2022; Qader et al., 2025b).

Chlorophyll extraction procedure

Chlorophyll was extracted using four commonly used organic solvents, namely methanol, ethanol, acetone, and diethyl ether. For each solvent, 10 mL of algae culture was mixed with 5 mL of solvent in triplicate. The mixtures were incubated under varying temperature conditions (20 °C, 40 °C, and 60 °C) for two different extraction times, i.e., 5 and 10 minutes (Qader and Shekha, 2023a). Following incubation, the samples were centrifuged, and the chlorophyll content in the supernatant was quantified using a UV-Vis spectrophotometer (UV-Visible 1240; Tecator, Rodgau, Germany) at a wavelength of at 660 and 643 nm, based on the method described by Arnon (1949)as described in Becker (1994).

$$\text{Chlorophyll a + b} = (7.12 \times A660) + (16.8 \times A643)$$

Statistical Analysis

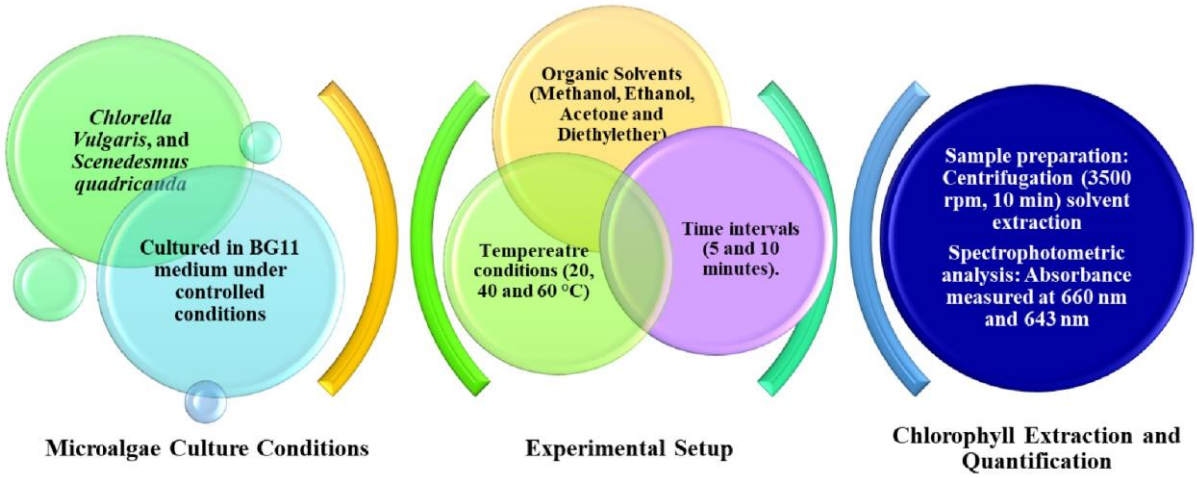

Fig. I. Workflow diagram of chlorophyll extraction and quantification from microalgae using organic solvents under varying experimental conditions (overview of the experimental workflow for chlorophyll extraction and quantification): *Chlorella vulgaris* and *Scenedesmus quadricauda* were cultured in BG11 medium under controlled conditions, subjected to different organic solvents (methanol, ethanol, acetone, and diethyl ether), extraction temperatures (20, 40, and 60 °C), and time intervals (5 and 10 minutes), followed by centrifugation and spectrophotometric analysis at 660 nm and 643 nm)

Data were analyzed using SPSS version 26.0. The normality of the data was assessed using the Shapiro-Wilk test. To evaluate the effects of solvent type, temperature, and extraction time on chlorophyll extraction, a two -way ANOVA (multi comparisons, Duncan test) was used to analyze the data and determine significant differences between time, temperature, and solvents. Means were compared the means at the significant level of P≤0.05 and Post hoc-Tukey test ($p<0.05$) was performed for pairwise comparisons between solvent groups (Pagano and Gauvreau, 2018). Additionally, indices such as EEI, TSI, TEI, CV, and CEI were calculated to assess the efficiency of the extraction methods comprehensively.

Results

Chlorophyll extraction from Chlorella vulgaris

The extraction efficiency of chlorophyll from Chlorella vulgaris varied significantly with changes in solvent, temperature, and extraction time (Table 1). At 20 °C, the methanol extraction in 5 minutes resulted in 12.3 ± 0.5 mg/L, which was significantly higher compared to ethanol (11.8 ± 0.4 mg/L), acetone (10.1 ± 0.3 mg/L), and diethyl ether (8.5 ± 0.2 mg/L). while after 10 minutes, methanol continued to show higher performance than the other solvents with 13.5 ± 0.6 mg/L, followed by ethanol (12.6 ± 0.5 mg/L), acetone (11.0 ± 0.4 mg/L), and diethyl ether (9.3 ± 0.3 mg/L). On the other hand, at 40 °C, the chlorophyll concentration from methanol reached 16.2 ± 0.7 mg/L at 5 minutes, significantly higher than ethanol (15.1 ± 0.6 mg/L), acetone (13.4 ± 0.5 mg/L), and diethyl ether (10.9 ± 0.4 mg/L). This trend continued at 10 minutes, where methanol led with 17.8 ± 0.8 mg/L, followed by ethanol (16.3 ± 0.7 mg/L), acetone (14.2 ± 0.6 mg/L), and diethyl ether (11.6 ± 0.5 mg/L). Moreover, at 60 °C, methanol extraction at 5 minutes resulted in 19.6 ± 0.9 mg/L, significantly higher than all other solvents, with ethanol extracting 18.2 ± 0.8 mg/L, acetone 16.0 ± 0.6 mg/L, and diethyl ether 13.1 ± 0.5 mg/L. After 10 minutes, the methanol extraction reached 21.0 ± 1.0 mg/L, significantly higher than ethanol (19.8 ± 0.9 mg/L), acetone (17.2 ± 0.7 mg/L), and diethyl ether (14.0 ± 0.6 mg/L).

Chlorophyll extraction from *Scenedesmus quadricauda*

Chlorophyll extraction from *Scenedesmus quadricauda* followed similar trends (Table 2), with methanol being the most effective solvent. At 20 °C, after 5 minutes, methanol extracted 10.8 ± 0.4 mg/L, significantly more than ethanol (10.2 ± 0.3 mg/L), acetone (8.9 ± 0.3 mg/L), and diethyl

Table 1
Chlorophyll extraction results of *Chlorella vulgaris* data represented as (means ± SE)

Temperature (°C)	Time (min)	Methanol (mg/L)	Ethanol (mg/L)	Acetone (mg/L)	Diethyl Ether (mg/L)
20	5 min	12.3 ± 0.5[a]	11.8 ± 0.4 [a]	10.1 ± 0.3[b]	8.5 ± 0.2[c]
	10 min	13.5 ± 0.6 [a]	12.6 ± 0.5 [b]	11.0 ± 0.4[c]	9.3 ± 0.3[d]
40	5 min	16.2 ± 0.7 [a]	15.1 ± 0.6[b]	13.4 ± 0.5[c]	10.9 ± 0.4[d]
	10 min	17.8 ± 0.8 [a]	16.3 ± 0.7 [b]	14.2 ± 0.6[c]	11.6 ± 0.5[d]
60	5 min	19.6 ± 0.9 [a]	18.2 ± 0.8 [b]	16.0 ± 0.6[c]	13.1 ± 0.5[d]
	10 min	21.0 ± 1.0 [a]	19.8 ± 0.9 [b]	17.2 ± 0.7[c]	14.0 ± 0.6[d]

Table 2
chlorophyll extraction of *Scenedesmus quadricauda* data represented as (Mean ± SE)

Temperature (°C)	Time (min)	Methanol (mg/L)	Ethanol (mg/L)	Acetone (mg/L)	Diethyl Ether (mg/L)
20°C	5 min	10.8 ± 0.4[a]	10.2 ± 0.3[a]	8.9 ± 0.3[b]	7.5 ± 0.2 [c]
	10 min	11.9 ± 0.5 [a]	11.0 ± 0.4[b]	9.8 ± 0.3[c]	8.1 ± 0.2[d]
40°C	5 min	14.5 ± 0.6 [a]	13.6 ± 0.5[b]	11.8 ± 0.4[c]	9.7 ± 0.3[d]
	10 min	16.1 ± 0.7 [a]	14.9 ± 0.6[b]	12.6 ± 0.5[c]	10.3 ± 0.4[d]
60°C	5 min	18.0 ± 0.8 [a]	16.8 ± 0.7[b]	14.5 ± 0.6 [c]	11.8 ± 0.5[d]
	10 min	19.4 ± 0.9 [a]	18.2 ± 0.8[b]	15.7 ± 0.7 [c]	12.6 ± 0.6[d]

ether (7.5 ± 0.2 mg/L). After 10 minutes, methanol yielded 11.9 ± 0.5 mg/L, again significantly higher than ethanol (11.0 ± 0.4 mg/L), acetone (9.8 ± 0.3 mg/L), and diethyl ether (8.1 ± 0.2 mg/L). Besides, at 40 °C, methanol at 5 minutes produced 14.5 ± 0.6 mg/L, significantly higher than the other solvents. The extraction increased to 16.1 ± 0.7 mg/L after 10 minutes, with ethanol extracting 14.9 ± 0.6 mg/L, acetone 12.6 ± 0.5 mg/L, and diethyl ether 10.3 ± 0.4 mg/L. Moreover, at 60 °C, methanol at 5 minutes resulted in 18.0 ± 0.8 mg/L, followed by ethanol (16.8 ± 0.7 mg/L), acetone (14.5 ± 0.6 mg/L), and diethyl ether (11.8 ± 0.5 mg/L). After 10 minutes, the chlorophyll concentration from methanol reached 19.4 ± 0.9 mg/L, which was significantly higher than the other solvents, with ethanol at 18.2 ± 0.8 mg/L, acetone at 15.7 ± 0.7 mg/L, and diethyl ether at 12.6 ± 0.6 mg/L.

ANOVA results for both *C. vulgaris* and *S. quadricauda* indicated significant effects of solvent type, temperature, and time on chlorophyll extraction as shown in (Table 3). For both algae species, solvent type had a highly significant impact on chlorophyll extraction, with F-values of 18.42 ($p<0.001$) for C. vulgaris, and 16.85 ($p<0.001$) for *S. quadricauda*. Additionally, temperature showed a significant effect on extraction efficiency, with F-values of 22.57 ($p<0.001$) for C. vulgaris and 20.91 ($p<0.001$) for *S. quadricauda*. Time had a significant effect on extraction, with F-values of 9.63 ($p=0.003$) for C. vulgaris and 8.74 ($p=0.004$) for *S. quadricauda*. The interaction between solvent and temperature was significant for both algae species, with F-values of 4.88 ($p=0.012$) for C. vulgaris and 4.22 ($p=0.016$) for *S. quadricauda*. Similarly, the interaction between solvent and time was significant for both species, with F-values of 3.71 ($p=0.025$) for C. vulgaris and 3.39 ($p=0.031$) for *S. quadricauda*. However, the interaction between temperature and time was not significant for either species, with F-values of 2.54 ($p=0.089$) for C. vulgaris and 2.31 ($p=0.105$) for *S. quadricauda*, indicating that the combined effect of temperature and time on chlorophyll extraction is minimal.

Post-hoc comparisons test revealed several important differences in extraction efficiency between solvents for both species. For *Chlorella vulgaris*, methanol showed significantly higher extraction efficiency compared to acetone (mean difference: 3.5, $p<0.001$) and diethyl ether (mean difference: 5.4, $p<0.001$). Ethanol resulted in significantly better extraction than acetone (mean difference: 2.3, $p<0.009$) and diethyl ether (mean difference: 4.2, $p<0.001$). The comparison between acetone and diethyl ether showed that acetone was more efficient (mean difference: 1.9, $p<0.03$), while for *Scenedesmus quadricauda*, methanol recorded significantly higher extraction

Table 3
Statistical results for *Chlorella vulgaris* and *Scenedesmus quadricauda*

Factor	*Chlorella vulgaris*		*Scenedesmus quadricauda*	
	F-value	p-value	F-value	p-value
Solvent type	18.42	<0.001	16.85	<0.001
Temperature (20, 40, 60°C)	22.57	<0.001	20.91	<0.001
Time (5, 10 minutes)	9.63	0.003	8.74	0.004
Interaction (Solvent × Temp)	4.88	0.012	4.22	0.016
Interaction (Solvent × Time)	3.71	0.025	3.39	0.031
Interaction (Temp × Time)	2.54	0.089	2.31	0.105

Table 4
Post-hoc Tukey Test for *Chlorella vulgaris* and *Scenedesmus quadricauda*

Comparison	*Chlorella vulgaris*		*Scenedesmus quadricauda*	
	Mean Difference	p-value	Mean Difference	p-value
Methanol vs Ethanol	1.2	0.045	1	0.052
Methanol vs Acetone	3.5	<0.001	3.1	0.002
Methanol vs Ether	5.4	<0.001	4.8	<0.001
Ethanol vs Acetone	2.3	0.009	2.1	0.018
Ethanol vs Ether	4.2	<0.001	3.8	<0.001
Acetone vs Ether	1.9	0.03	1.7	0.045

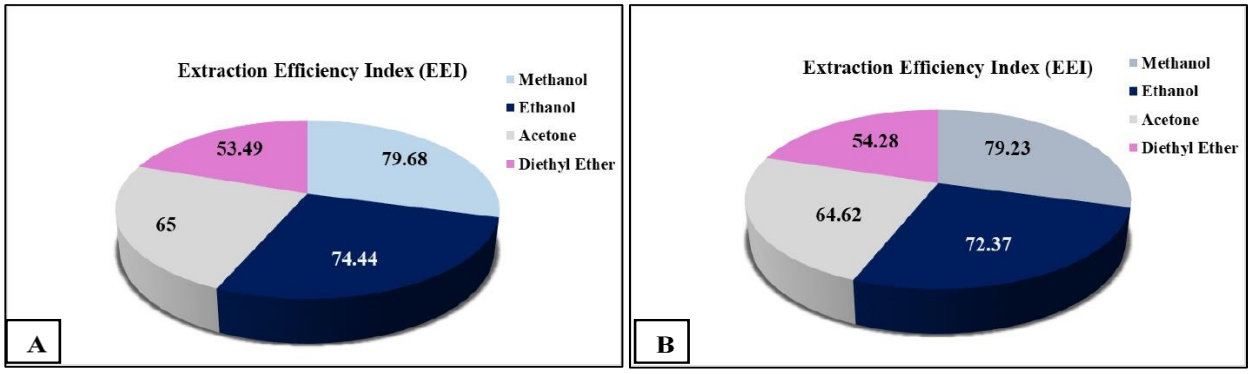

Fig. II. Extraction Efficiency Index values of (A) *Chlorella vulgaris*, (B) *Scenedesmus Quadricauda*

efficiency than acetone (mean difference: 3.1, p<0.002) and diethyl ether (mean difference: 4.8, p<0.001). Ethanol performed similar to methanol but showed slightly lower efficiency, with significant differences being observed compared to acetone (mean difference: 2.1, p<0.018) and diethyl ether (mean difference: 3.8, p<0.001). On the other hand, acetone was found to be more effective than diethyl ether (mean difference: 1.7, p<0.045). These results further support the higher performance of methanol and ethanol for chlorophyll extraction from *Scenedesmus quadricauda,* with methanol being the most efficient solvent (Table 4).

Indices for chlorophyll extraction efficiency

Extraction Efficiency Index (EEI)

Concerning the Extraction Efficiency Index of *Chlorella vulgaris*, methanol exhibited the highest EEI value of 79.68, followed by ethanol (74.44), acetone (65.00), and diethyl ether (53.49). These values confirmed that methanol was the most efficient solvent for chlorophyll extraction in *C. vulgaris*, significantly outperforming the other solvents. A similar pattern was observed for *S. quadricauda*, where methanol showed the highest EEI value (79.23), followed by ethanol (72.37), acetone (64.62), and diethyl ether (54.28) (Fig. II).

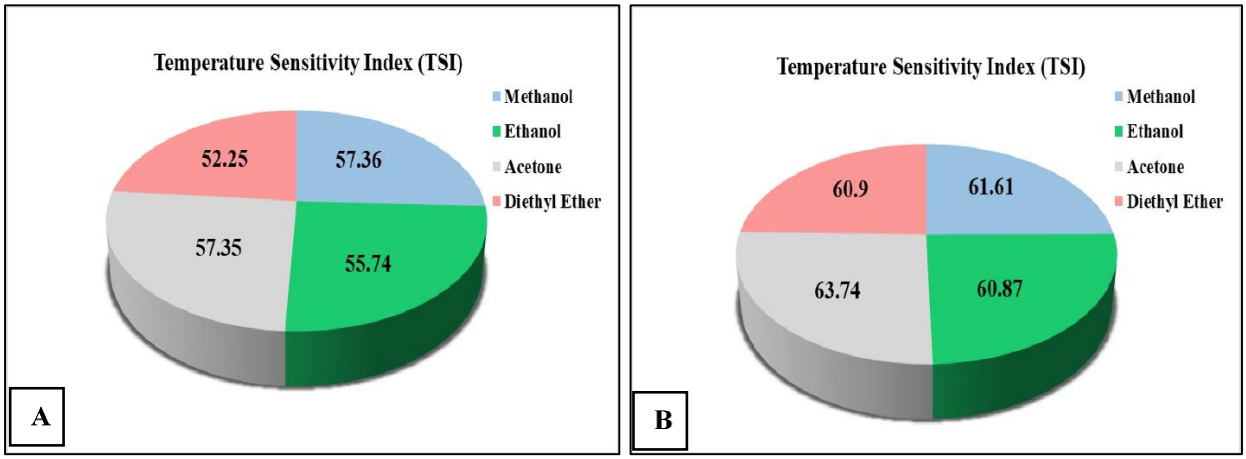

Fig. III. Temperature Sensitivity Index Values of (A) *Chlorella vulgaris* (B) *Scenedesmus Quadricauda*

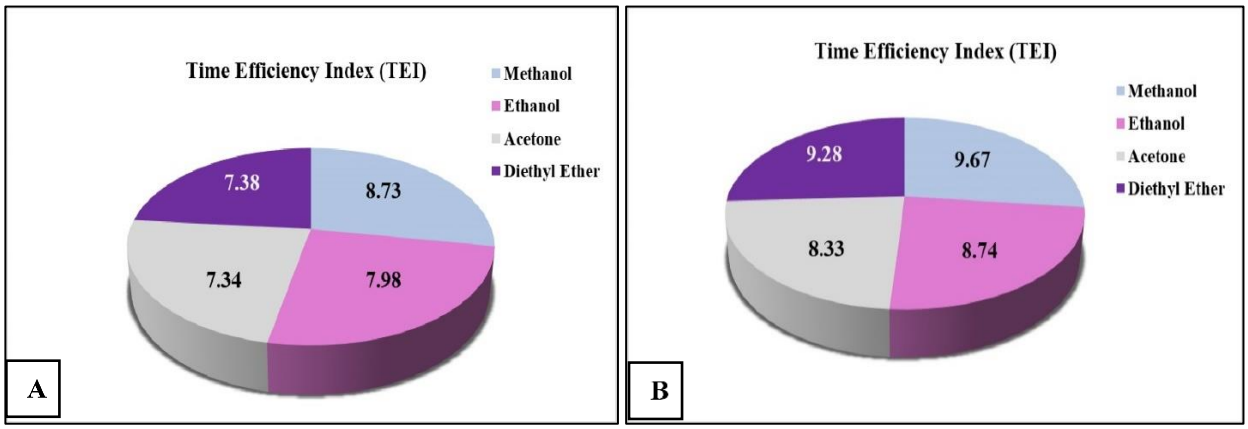

Fig. IV. Time Efficiency Index of (A) *Chlorella vulgaris* (B) *Scenedesmus Quadricauda*

Temperature Sensitivity Index (TSI)

The Temperature Sensitivity Index of *Chlorella vulgaris*, using methanol as solvent showed a TSI of 57.36, indicated a high temperature sensitivity. Ethanol (TSI=55.74) and acetone (TSI=57.35) demonstrated very similar temperature sensitivity, whereas diethyl ether exhibited the lowest TSI (52.25), indicating relatively low sensitivity to temperature variations. Similarly, in *Scenedesmus quadricauda*, methanol showed the highest TSI (61.61), followed by ethanol (60.87), acetone (63.74), and diethyl ether (60.9) (Fig. III).

Time Efficiency Index (TEI)

The time efficiency index (TEI) provided insight into the time-dependent efficiency of the solvents (Fig. IV). For Chlorella vulgaris, methanol had a TEI of 8.73, indicating that longer extraction times led to a considerable increase in chlorophyll yield. Ethanol had a TEI of 7.98, acetone had 7.34, and diethyl ether had 7.38, demonstrating that methanol was the most time-efficient solvent for chlorophyll extraction. In *Scenedesmus quadricauda*, methanol achieved a TEI of 9.67. TEI of ethanol, acetone, and diethyl ether were 8.74, 8.33, and 9.28, respectively.

Coefficient of Variation (CV)

The coefficient of variation (CV) results for *C. vulgaris* using methanol showed a CV value of 20.35, indicating moderate variation in extraction efficiency. Ethanol had a CV of 19.94, acetone 20.23, and diethyl ether 18.92, with diethyl ether demonstrating the lowest variation among the solvents. For *S. quadricauda*, the CV values were slightly higher: methanol (21.51) showed the highest variation, followed by ethanol (21.32), acetone (21.95), and diethyl ether (21.50) (Fig. V).

Comprehensive Extraction Index (CEI)

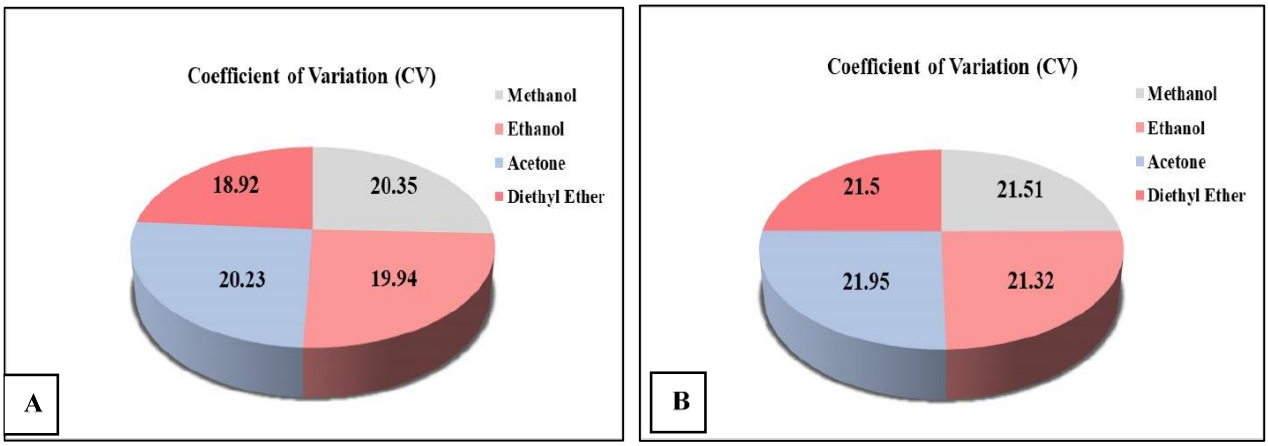

Fig. V. Coefficient of Variation of (A) *Chlorella vulgaris* (B) *Scenedesmus Quadricauda*

he Comprehensive Extraction Index (CEI) results for *Chlorella vulgaris* showed that methanol achieved the highest CEI value of 3.06, followed by acetone (2.81), ethanol (2.69), and diethyl ether (2.68). Similarly, for *Scenedesmus quadricauda*, methanol again had the highest CEI value of 2.69, followed by diethyl ether (2.79), acetone (2.73), and ethanol (2.66) (Fig. VI).

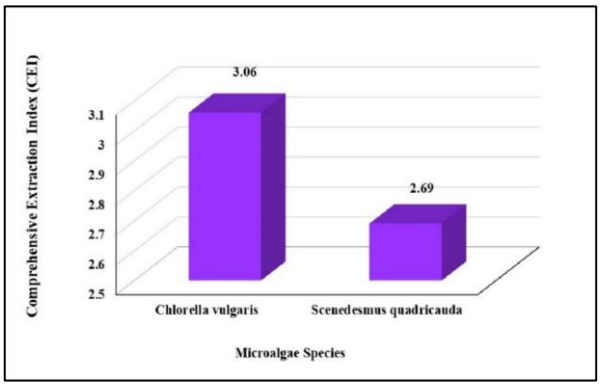

Fig. VI. Comparison of Comprehensive Extraction Index (CEI) in *Chlorella vulgaris* and *Scenedesmus quadricauda*

Discussion

The results of the study demonstrated the higher efficacy of methanol for chlorophyll extraction from *Chlorella vulgaris* and *Scenedesmus quadricauda*. The significant differences in chlorophyll extraction between solvents were similar with previous research reporting that methanol is often the most effective solvent for chlorophyll extraction in various algal species. The studies by Amin et al. (2018)and Varaprasad et al. (2019)reported that methanol was able to extract chlorophyll from *Chlorella vulgaris* more efficiently than both ethanol and acetone. Similarly, (Pechar, 1987) found that methanol extracted chlorophyll in higher concentrations compared to ethanol and acetone from Scenedesmus sp. Singh et al. (2020), reported similar results, where methanol provided higher chlorophyll yields due to its strong polar characteristics, which aids in the dissolution of chlorophyll molecules. In comparison, ethanol, while less effective than methanol, still showed reasonable extraction yields. In their study, Yu et al. (2024) showed that ethanol was relatively effective for chlorophyll extraction, but generally not as efficient as methanol. Ethanol is a preferred choice when environmental sustainability and reduced toxicity are priorities, as it is less harmful to both the algae and human health compared to methanol. Ethanol has also been reported as an effective solvent for chlorophyll extraction, especially in non-toxic or eco-friendly applications (Jorge et al., 2024). Similarly, Varaprasad et al. (2021)reported that ethanol produced moderate chlorophyll yields from green microalgae. Acetone and diethyl ether showed the least chlorophyll extraction. From the studies of Varaprasad et al. (2019), acetone proved to be less effective as a chlorophyll extracting from *Chlorella vulgaris* when compared to ethanol and methanol. Moreover, similar to the findings of Varaprasad et al. (2019), it was found in the present study that acetone was less effective for chlorophyll extraction due to its inability to disrupt algal cell walls as efficiently as methanol and ethanol. According to the previous studies, time and temperature significantly impact chlorophyll

extraction with higher temperatures (60 °C) and long extraction time (10 min) providing better yield compared to chlorophyll extraction at low temperatures and short extraction time (Georgiopoulou et al., 2023; Ngcobo et al., 2024); it was also found that diethyl ether tends to produce lower chlorophyll extraction results compared to methanol, ethanol, and acetone. The weak polar nature of diethyl ether limits its ability to solvate chlorophyll molecules, making it less suitable for efficient extraction. Fabrowska et al. (2018)and Wang et al. (2024a) reported similar trends for other algae species.

Conclusion

This study demonstrated that methanol exhibited the highest extraction efficiency for both *Chlorella vulgaris* and *Scenedesmus quadricauda*. Maximum chlorophyll yields were recorded after 10 minutes of extraction at 60 °C, reaching 21.0 mg/L for C. vulgaris and 19.4 mg/L for *S. quadricauda*. Methanol also had the highest scores in extraction indices such as the Extraction Efficiency Index (EEI), Temperature Sensitivity Index (TSI), and Comprehensive Extraction Index (CEI). Statistical analyses confirmed significant effects ($p<0.001$) of solvent type, temperature, and time on extraction performance, with methanol showing superior efficiency over ethanol, acetone, and diethyl ether. Ethanol, while less effective than methanol, remains a viable alternative in eco-friendly and non-toxic extractions. Acetone and diethyl ether proved to be less efficient, particularly under higher temperatures. According to the results, methanol is recommended as the solvent of choice for large-scale chlorophyll extraction from *Chlorella vulgaris* and *Scenedesmus quadricauda*, with implications for both laboratory and industrial applications.

Availability of data and materials

All data and materials supporting the findings of this study are available from the corresponding author upon reasonable request.

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