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Pretreatment strategy for enhanced lipid extraction from algal biomass in bio-oil production

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Efficient lipid extraction from algal biomass is a critical step in bio-oil production. Optimizing extraction parameters has led to the development of various methodologies; however, the primary challenge remains in effectively disrupting algal cells to facilitate lipid release. This study evaluates the impact of different pretreatment techniques on traditional lipid extraction methods, focusing on lipid extraction efficiency. Pretreatment methods examined include physical disruption (blending), irradiation (microwaving), and a novel combination of both. Traditional lipid extraction methods, such as Bligh-Dyer, Folch, and Soxhlet are employed. Results demonstrated that lipid extraction efficiency increased from 2.22–22.12% of dry weight (untreated biomass) to 10.09–52.08% of dry weight (pretreated biomass), varying across different algal species. The combined pretreatment (blender and microwave) followed by the Bligh-Dyer method yielded the highest lipid recovery. *Chlamydomonas reinhardtii* showed the maximum lipid content (52.08±2.22%). These findings highlight the significant enhancement in lipid recovery using the novel pretreatment and suggest its potential for improving bio-oil production.

Keywords: Algal oil, Biomass processing, Cell disruption, Lipid extraction, Lipid productivity

Introduction

Algae are promising candidates for bio-oil production due to their renewable nature and ability to generate value-added products. They exhibit high growth rates, photosynthetic efficiency, stress tolerance, and nutrient uptake capabilities[1]. With a higher lipid concentration compared to conventional oil crops, algae can produce 15 to 300 times more oil than traditional sources[2]. Algal species are categorized based on their lipid content, which can be enhanced by cultivating them under specific physiological conditions. However, the major challenges include low biomass yields and complex lipid extraction processes. High downstream processing costs, especially at an industrial scale, present a significant bottleneck[3]. Lipid extraction efficiency is largely dependent on the lipid content of the algae, which varies among species and is influenced by growth conditions.

Algae exhibits an ability to adapt rapidly to changing physiological conditions, modulating lipid composition through *de novo* synthesis and fatty acid recycling, which maintains membrane integrity. Lipid accumulation tends to increase during slower metabolic rates, especially under nutrient stress[4]. Therefore, the present study selected algal cultures with varying lipid content to maximize the effect of different pretreatment methods across species.

Since lipids are stored within the algal cell wall, efficient cell disruption is a key step in lipid extraction[5]. The thick algal cell walls present a barrier, necessitating effective disruption techniques[6]. After cell harvesting, traditional lipid extraction methods, including Bligh-Dyer, Folch, and Soxhlet, are commonly employed, but these methods can be time-consuming and may contaminate the residual biomass, limiting further applications[7]. Innovative techniques have been developed to overcome these challenges, such as releasing free fatty acids from bound lipids either before or after extraction. Additionally, wet disruption methods using ethanol as a safe solvent, and dilute acid pretreatment to hydrolyze carbohydrates, have shown promise in enhancing lipid recovery[8]. The techniques had some limitations such as in case of release of free fatty acids from bound lipids, releasing free fatty acids, generally, does not achieve complete conversion, especially when the bound lipids are found in

complex forms. This condition makes the methods; energy intensive, which either require high temperatures or specialized enzymes, leading to increased input and cost. In wet disruption method, while ethanol is safe solvent but has limited solubility. This leads to selective extraction and lower yield. In case of dilute acid pretreatment for hydrolysis, it increases the risk of producing inhibitory byproducts such as furfurals, contaminating the extracted lipid.

These limitations instigated the search of finding methods which focused on algal species composition and interactions between lipid and non-lipid components after extraction efficiency. There was a need to fulfill the gap of studies on combining techniques for enhanced production, highlighting the impact of extraction methods on refining processes.

Hence, this study investigated the improvement of lipid extraction efficiency using various pretreatment methods on six algal species with different lipid contents. The selected species represented a diverse range of lipid accumulation and biomass productivity profile. *Chlamydomonas reinhardtii*, *Chlorella pyrenoidosa*, and *Chlorella vulgaris* are well established species and hence can be considered as benchmark (positive controls) for comparison. Also, as they are known for their environmental adaptability, they are reliable for reproducibility. *Chlorella sorokiniana*, *Scenedesmus abundans*, and *Scenedesmus obliquus* have been reported but less explored species for lipid productivity. The selected species are also known to grow under a variety of environmental conditions including light intensity and temperature. Conclusively, this study of six different species will ensure the findings are robust and help in finding the best possible species and scenario for lipid extraction. Algal biomass was subjected to physical disruption (blender), irradiation (microwave), and a novel combination of both methods. The pretreated biomass was then subjected to modified versions of traditional lipid extraction methods to compare lipid recovery and productivity. The effect of each pretreatment was assessed in combination with all extraction methods, and the best results were validated across all species selected for the study.

Experimental Section

Algae species and growth conditions

Algal species namely *Chlamydomonas reinhardtii*, *Chlorella pyrenoidosa, Chlorella sorokiniana, Chlorella vulgaris* and *Scenedesmus abundans* were procured from the Indian Institute of Technology, Guwahati, India and *Scenedesmus obliquus* was procured from the National Collection of Industrial Microorganisms, Pune, India. The cultures were grown in BG-11 medium at temperature of 27.0±2 ℃. The volume of the flask was 2L, with a working volume of culture as 1L. The pH was adjusted to 7.2 at initial. The cultures were illuminated with cool white, fluorescent lamps (SYSKA, T5 – 4W – 0.M – 6500K) of 200 μmol m^{-2} s^{-1} ensuring a consistent photoperiod of 14 h light: 10 h dark cycles. Growth of all species was monitored by measuring the optical density at 680 nm (OD_{680}).

Biomass production

Algal cultures were grown in triplicates and samples were withdrawn on the 3rd, 6th, 9th, 12th, and 15th day, respectively. The harvested wet biomass was centrifuged at 6500 rpm (~4500 g) for 10 min and the pellets so formed were isolated and washed with phosphate buffer solution followed by overnight drying at 65℃ in a hot air oven. The dried algal biomass obtained was then stored at -20℃ for further analysis. The biomass productivity was estimated from the dry weight of algal cultures as per the protocol reported by Hempel et al., 2012[9], while the Dry Cell Weight (DCW) was measured by the final dry weight (g) of algae per unit volume of culture (L). The biomass concentration of algae was calculated from equation [Eq. (1)].

$$Biomass\ concentration\ of\ algae\ (g\ L^{-1}) = \frac{Final\ dry\ weight(g)}{Volume\ of\ culture\ (L)} \quad \ldots (1)$$

Lipid accumulation

For lipid accumulation profiling, 50 milligrams of dried algae samples was used to measure lipid content on the 3rd, 6th, 9th, 12th, and 15th day, respectively. All the samples were taken in triplicates. The Bligh-Dyer method, without pretreatment of algal biomass, was used for lipid extraction.

Pretreatment of algal biomass

For pretreatment, 1 g (DCW) of algal biomass was mixed with 1 mL of distilled water and thereafter subjected to the following cell disruption protocols:

Blender (B)

The volume of the sample was made up to 100 mL using distilled water. The sample was subjected to shear disruption using a blender (Model No. HAMG600w15, Borosil) with a double blade operating at 20,000 rpm for 5 min in a 0.4 L heavy gauge, stainless steel, leak-proof jar. The process was repeated thrice at an interval of 1 min and average volume was reported.

Microwave (M)

The sample volume was increased to 10 mL by adding distilled water and digested in the microwave (Model No. 20BC4, IFB) with irradiation of 2,450 MHz and 800 W output power. The temperature was raised to 100°C and maintained for 5 min followed by cooling (to stop the spillage of the sample inside the microwave). Any loss of water during the heating process was compensated by the addition of distilled water.

Combination Blender and Microwave (CBM)

The algal sample was blended for 1 min and then subjected to heating in the microwave for 1-2 min till it boils at 100°C. Loss of sample volume was made up by distilled water after microwave and same volume was maintained before reinitiating the process. The above process was performed twice to ensure homogeneity of the sample. By this, the localized overheating was minimised. It ensured uniform heating and avoided thermal degradation. However, the overall time for the cells to encounter microwave remains same.

Lipid Extraction and Estimation

Traditional lipid extraction protocols (Bligh-Dyer, Folch, and Soxhlet methods) are typically the economical and classical examples of easy-to-use methods.

Bligh-Dyer Method

The lipid extraction from the dried algal biomass was performed by Bligh-Dyer Method[10]with minor modifications, which featured addition of 100 mg dry biomass in 20 mL distilled water followed by its mixing with chloroform and methanol in a ratio of 1:1:2. The mixture was thereafter blended for 1 min and kept for 4 h. This was followed by the addition of 4 mL of pure water for phase separation. After 2-3 h organic phase of the sample, containing lipids, was separated by pipetting it carefully. The organic layer was then filtered through glass fibre filter paper and the sample was stored in a pre-weighed aluminium cup. The resultant solution was dried overnight at 60 °C using a hot air oven and the lipid content was calculated by the equation [Eq. (2)][11].

$$Lipid(mg) = \frac{F_1(mg) - F_2(mg)}{V_a(mL)} xV(mL). \quad \ldots (2)$$

where F_1 and F_2 were the weight of the aluminium cup before and after drying; V_a and V were the volumes of the aqueous phase and organic phase of the sample, respectively.

Folch Method

Folch method[12] with slight modifications was used in the present investigation, for lipid extraction wherein 1 g of dried algal powder was added in 10 mL of chloroform: methanol solution (ratio of 2:1). The solvent mixture was then mixed in a cyclo-mixer for 10 min followed by its incubation for 24 h to facilitate the phase separation. The transparent layer containing lipids was thereafter filtered and transferred to a pre-weighed aluminium cup. The solvent was thereafter evaporated using a rotary evaporator and the lipid content was quantified gravimetrically. This protocol was repeated thrice, and the average values were reported. The lipid content was calculated by the equation [Eq. (3)].

Soxhlet Method

The sample, 50 g of dried algae powder, was placed in a thimble and solvent (n-hexane) was added to the round bottom flask in the Soxhlet extraction apparatus. The extraction was initiated by heating the flask at 65°C for ~ 60 solvent recycles in 3 h wherein the rising of the vapour, its condensation, and the dripping back of the solvent onto the sample flask featured a single reflux cycle. After 24 h, hexane extracts (the oil from the algal biomass), and the crude oil were separated by evaporating the hexane in a rotary evaporator[13]. The lipid content was calculated by the equation [Eq. (3)].

Lipid content (C) was measured gravimetrically and quantified by Eq. (3) as percentage of dry cell weight[14].

$$C = \frac{W_1 - W_2}{W_1} x100 \quad \ldots (3)$$

where W_1 and W_2 were the weight of algal biomass before and after lipid extraction, respectively.

Lipid productivity (P) was estimated by Eq. 4[14]

$$P = \frac{Lipid\ content\ (\%\ of dry\ weight)\ x\ biomass\ (g\ L^{-1})}{Cultivation\ period\ (day)} \quad \ldots (4)$$

Statistical Analysis

Data processing and analysis was performed by statistical software (Graph Pad Prism 9.5.1). Two-way ANOVA analysis was applied on the data representing the total percentage of lipid content extracted by different protocols followed by Tukey multiple comparisons with $P<0.05$ as the significant value. All the experimental data were reported as Mean ± Standard Error Mean as it quantifies uncertainty in the estimate of the mean.

Results and Discussion

Results

Growth Measurement

The inoculum used for algal growth was used from exponential growth stage, therefore the adaptation phase is not considered in the current study. The stationary phase was observed after 12th day in all the algal species. Results showed in Table 1 indicated that growth of all the species varied drastically among different species, with accumulation of biomass for the six species ranged from 1.27±0.2 g $L^{-1}$to 5.42±0.88 g L^{-1}. The maximum biomass was produced by *Scenedesmus obliquus* at the 15th day, with the value 6.3 g L^{-1}.

Lipid analysis

Lipid content

Algal species under experimentation showed similar lipid accumulation patterns. It was shown in Fig. 1 that the lipid content increased as the biomass started increasing and attained the maximum magnitude after 12 days of cultivation. However, the rate of lipid accumulation varied vividly among species depending on the inherent characteristics of algal species.

The present study also showed that species belonging to similar species (*Chlorella pyrenoidosa*, *Chlorella sorokiniana*, etc.) bears different growth and lipid accumulation patterns throughout experiment. The maximum lipid content was observed in case *Chlamydomonas reinhardtii* followed by *Chlorella pyrenoidosa* with total lipid content of

Table 1 — Dry Biomass of six algal species at the end of end (15th day) of cultivation experiment

Algal species	Dry Biomass Conc. (g L^{-1})
Chlamydomonas reinhardtii	3.97±0.24
Chlorella pyrenoidosa	3.02±0.46
Chlorella sorokiniana	2.87±0.65
Chlorella vulgaris	1.27±0.2
Scenedesmus abundans	2.33±0.25
Scenedesmus obliquus	5.42±0.88

#Data shown as Mean ± Standard Error Mean

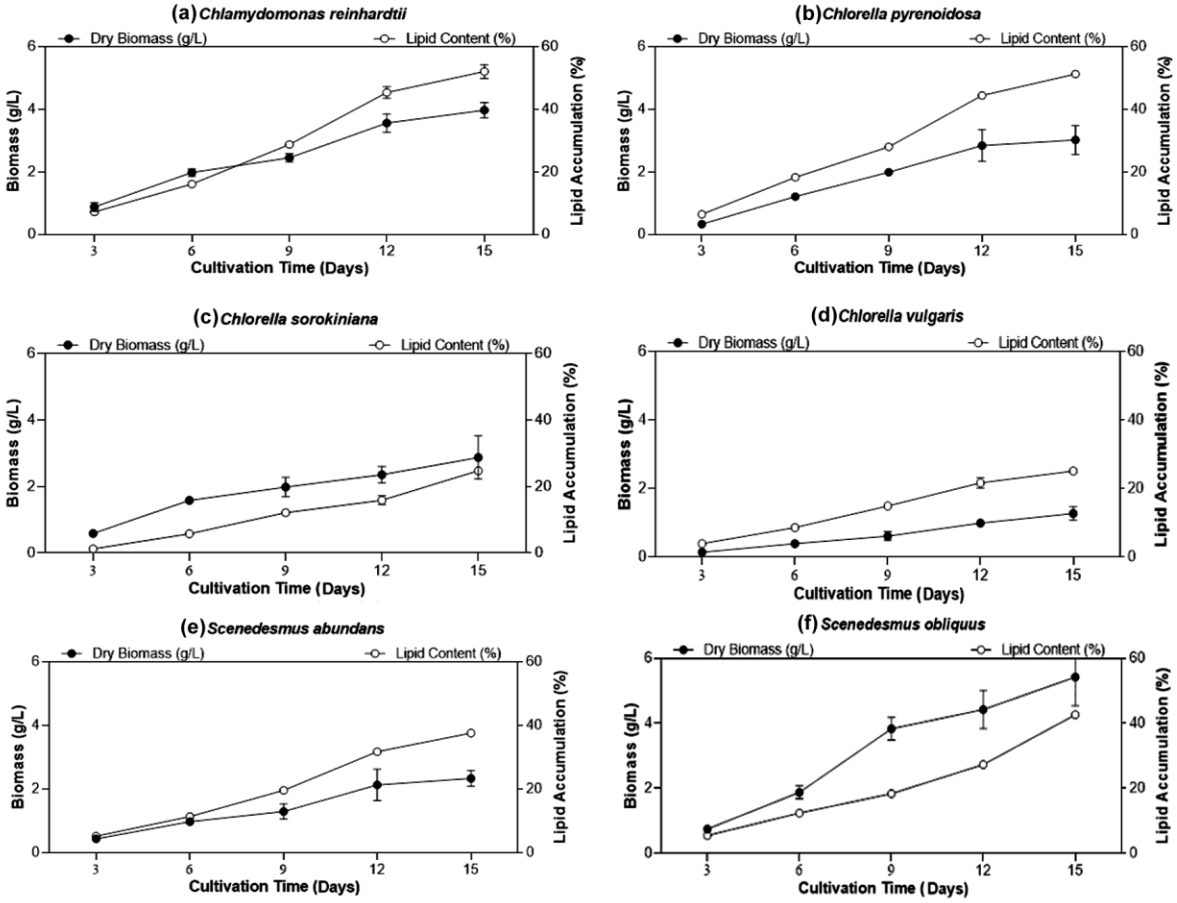

Fig. 1 — Dry biomass and lipid content at different cultivation times for (a) *Chlamydomonas reinhardtii*, (b) *Chlorella pyrenoidosa*, (c) *Chlorella sorokiniana*, (d) *Chlorella vulgaris*, (e) *Scenedesmus abundan* and (f) *Scenedesmus obliquus*

approximately 52% and 51%, respectively. These lipid content values used in Fig. 1 were extracted by using the best strategy, identified in the present study, to extract lipid from all algal species.

Lipid productivity

To identify the best algal oil producer species, lipid productivities of all the species were investigated. On comparison, it was observed that the highest lipid productivity occurred after 12th till 15th day, highlighting the best harvesting time for lipid extraction as 15th day under controlled conditions. Lipid productivity of algal species, under observation, ranged from 0.67 mg L^{-1} $d^{-1}$to 6.30 mg L^{-1} d^{-1}(Table 2).

Effect of pretreatment methods

The algal species after 15 days of cultivation were harvested and subjected to selected lipid extraction methods. The lipid content obtained, without pretreatment, ranged from 2.22±0.33% to 22.12±0.24%.

Table 2 — Lipid content and lipid productivity obtained with and without combinations of pretreatment methods followed by lipid extraction methods

Algal Species	Pre-Treatment	Extraction Method	Lipid Content (%)	Lipid Productivity (mg L^{-1} d^{-1})
Chlamydomonas reinhardtii	None	Bligh-Dyer	18.67±0.28	4.95±0.38
		Folch	17.65±0.47	4.68±0.36
		Soxhlet	11.43±0.51	3.03±0.29
	Blender	Bligh-Dyer	33.43±0.84	8.83±0.46
		Folch	32.21±0.56	8.53±0.6
		Soxhlet	27.70±0.59	7.34±0.55
	Microwave	Bligh-Dyer	39.91±1.11	10.58±0.82
		Folch	37.78±0.27	9.99±0.54
		Soxhlet	31.13±0.68	8.23±0.45
	Combination (CBM)	Bligh-Dyer	52.08±2.22	13.86±1.43
		Folch	38.56±0.44	10.21±0.68
		Soxhlet	25.78±0.08	6.82±0.43
Chlorella pyrenoidosa	None	Bligh-Dyer	13.65±0.23	2.76±0.46
		Folch	10.09±0.29	2.05±0.37
		Soxhlet	5.88±0.17	1.18±0.17
	Blender	Bligh-Dyer	35.67±0.55	7.15±0.97
		Folch	29.54±0.17	5.94±0.89
		Soxhlet	18.89±0.42	3.79±0.55
	Microwave	Bligh-Dyer	41.11±0.30	8.29±1.27
		Folch	34.20±0.35	6.90±1.09
		Soxhlet	20.08±0.18	4.04±0.59
	Combination (CBM)	Bligh-Dyer	51.28±1.20	10.25±1.32
		Folch	36.66±0.96	7.41±1.2
		Soxhlet	26.78±0.25	5.41±0.87
Chlorella sorokiniana	None	Bligh-Dyer	8.26±0.23	1.59±0.37
		Folch	5.56±0.21	1.08±0.28
		Soxhlet	2.22±0.33	0.44±0.15
	Blender	Bligh-Dyer	18.23±0.43	3.53±0.87
		Folch	16.29±0.53	3.09±0.68
		Soxhlet	11.67±0.46	2.19±0.42
	Microwave	Bligh-Dyer	22.56±0.16	4.32±0.98
		Folch	20.02±0.18	3.82±0.84
		Soxhlet	16.66±0.09	3.19±0.73
	Combination (CBM)	Bligh-Dyer	24.71±0.48	4.75±1.13
		Folch	23.19±0.31	4.42±0.96
		Soxhlet	16.78±0.06	3.22±0.74

(*Contd*.)

Table 2 — Lipid content and lipid productivity obtained with and without combinations of pretreatment methods followed by lipid extraction methods (*Contd*.)

Algal Species	Pre-Treatment	Extraction Method	Lipid Content (%)	Lipid Productivity (mg L^{-1} d^{-1})
Chlorella vulgaris	None	Bligh-Dyer	10.11±0.57	0.87±0.18
		Folch	9.76±0.13	0.82±0.13
		Soxhlet	7.21±0.27	0.61±0.09
	Blender	Bligh-Dyer	14.89±0.17	1.27±0.22
		Folch	12.11±0.57	1.04±0.21
		Soxhlet	10.09±0.54	0.86±0.16
	Microwave	Bligh-Dyer	22.94±0.43	1.95±0.35
		Folch	21.01±0.46	1.77±0.26
		Soxhlet	13.14±0.08	1.11±0.17
	Combination (CBM)	Bligh-Dyer	25.11±0.32	2.12±0.33
		Folch	22.94±0.45	1.95±0.34
		Soxhlet	18.11±0.34	1.54±0.26
Scenedesmus abundans	None	Bligh-Dyer	22.12±0.24	3.43±0.35
		Folch	20.10±0.57	3.11±0.31
		Soxhlet	17.98±0.19	2.79±0.28
	Blender	Bligh-Dyer	28.96±0.57	4.51±0.54
		Folch	24.28±0.12	3.78±0.42
		Soxhlet	19.90±0.32	3.09±0.3
	Microwave	Bligh-Dyer	37.50±0.21	5.82±0.6
		Folch	35.56±0.38	5.52±0.56
		Soxhlet	26.67±0.38	4.13±0.38
	Combination (CBM)	Bligh-Dyer	37.58±0.41	5.84±0.65
		Folch	35.79±0.80	5.55±0.54
		Soxhlet	31.10±0.39	4.82±0.45
Scenedesmus obliquus	None	Bligh-Dyer	17.32±0.69	6.30±1.2
		Folch	15.54±0.09	5.61±0.9
		Soxhlet	10.38±0.33	3.77±0.69
	Blender	Bligh-Dyer	18.90±0.26	6.80±1.01
		Folch	17.30±0.56	6.32±1.21
		Soxhlet	15.90±0.55	5.71±0.82
	Microwave	Bligh-Dyer	41.90±0.66	15.18±2.61
		Folch	36.21±0.77	13.04±2.03
		Soxhlet	33.45±1.41	11.92±1.45
	Combination (CBM)	Bligh-Dyer	42.60±1.06	15.45±2.65
		Folch	39.97±1.74	14.54±2.61
		Soxhlet	34.31±1.44	12.48±2.39

#Data shown as Mean ± Standard Error Mean

Afterwards, the dried biomass was subjected to different pretreatment and the lipid content so obtained was summarised in Table 2. The maximum lipid content was obtained in case of *Chlamydomonas reinhardtii* with a lipid content of 54.3% using combination of pretreatment and Bligh-Dyer method of lipid extraction. The lipid content obtained in *Chlamydomonas reinhardtii* without pretreatment using Bligh-Dyer method of lipid extraction was 18.67±0.28%. The impact of pretreatment on lipid extraction in this case can be calculated as increase of approximately 2.79folds. Similarly, maximum impact was seen in case of *Chlorella pyrenoidosa*, where the lipid content increased from 13.65% to 52.48%, computing an increase of 3.84 folds.

Significance of pretreatment method

A statistical comparison of lipid content obtained in Table 2 was performed and shown in Fig. 2. This comparison led to identify the impact of all the pretreatment methods on respective lipid extraction

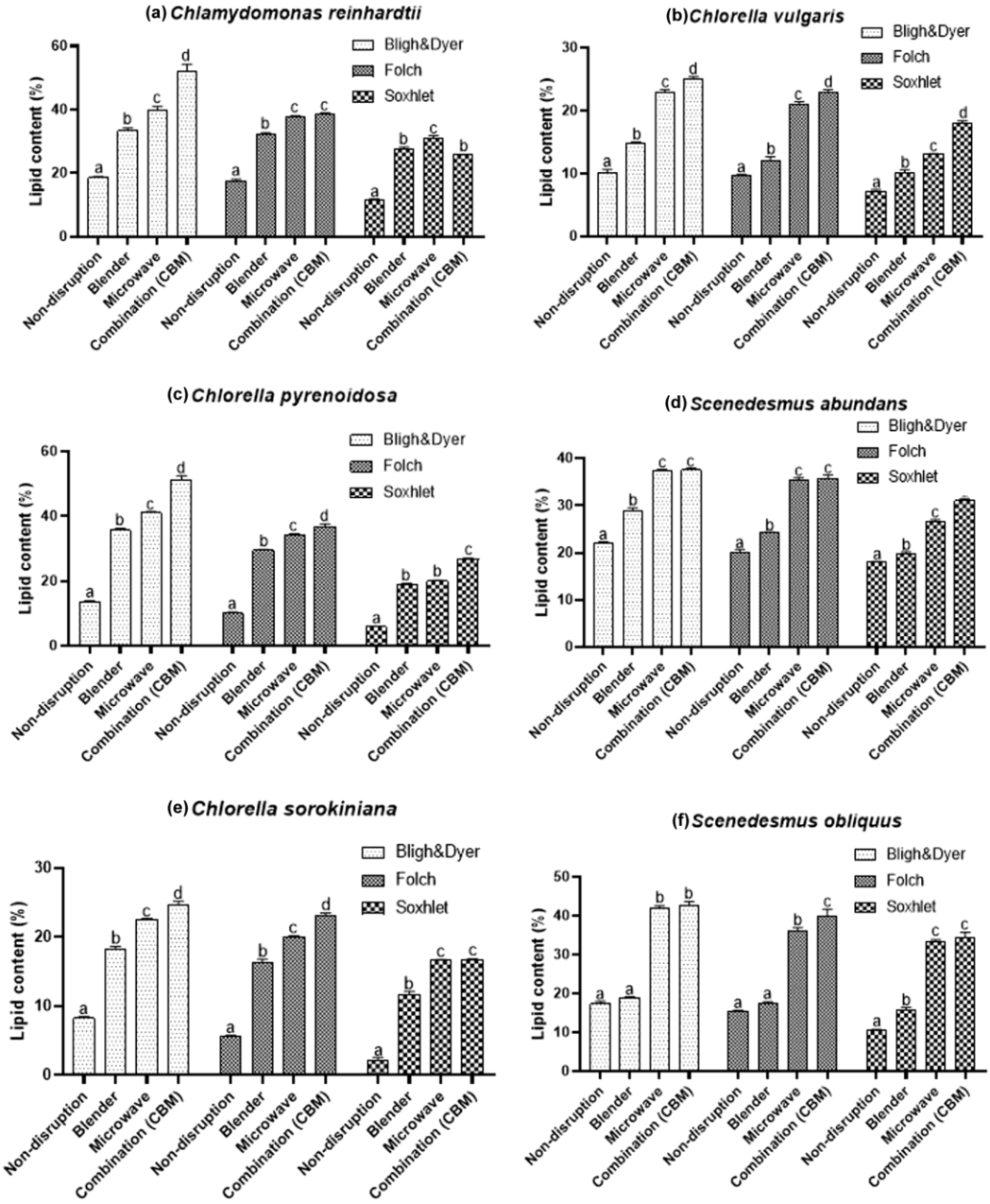

Fig. 2 — Response analysis of individual and combination of pretreatment and extraction protocols for different algal cultures. The graphs depict the significant ($P<0.05$) difference in lipid extraction methods (Bligh-Dyer, Folch and Soxhlet) with (Blender, Microwave and Combination) or without (non-disruption) pretreatment methods among species (a) *Chlamydomonas reinhardtii*, (b) *Chlorella pyrenoidosa*, (c) *Chlorella sorokiniana*, (d) *Chlorella vulgaris*, (e) *Scenedesmus abundans* and (f) *Scenedesmus obliquus*

method. The results showed that pretreatment has significantly contributed toward the enhancement of lipid extraction efficiency across all algal species. Also, the innovative pretreatment methods developed in the present study increased the lipid extraction efficiency with all the three lipid extraction methods in majority of the species.

Discussion

Algae are always considered as a potential source for feedstock for bio-oil production as they have faster growth rate and high lipid accumulation. This study displayed significant variation in biomass concentration and lipid content across different algal species over the course of the cultivation period, with

notable differences observed at specific time intervals. These variations highlighted the diverse metabolic responses of the algal species under study and suggested potential optimization strategies for algal lipid extractions with available biomass. The maximum lipid content (% dry wt.), observed for different algal species after 15 days of cultivation (Table 2) was as follows: *Chlamydomonas reinhardtii* (52.08±2.22), *Chlorella pyrenoidosa* (51.28±1.20), *Scenedesmus obliquus* (42.60±1.06), *Scenedesmus abundans* (37.58±0.41), *Chlorella vulgaris* (25.11±0.32) and *Chlorella sorokiniana* (24.71±0.48). It was also observed that for algal species with lower lipid content (*Scenedesmus abundans*, *Chlorella vulgaris,* and *Chlorella sorokiniana),* the lipid content increased with growing biomass. Moreover, *Chlamydomonas reinhardtii* and *Chlorella pyrenoidosa* exhibited higher lipid accumulation as opposed to their respective biomass yield, thereby emerging as potential algal species for algal lipid production. In-depth analysis was done for the biomass and lipid accumulation (Fig. 1) for the different algal species in the present investigation. It was observed that *Chlamydomonas reinhardtii* exhibited a faster growth rate and biomass accumulation of 0.88±0.14 g L^{-1} whereas *Chlorella vulgaris* featured slowest growth rate & biomass accumulation of (0.14±0.01 g L^{-1}) in 3 days. However, the maximum biomass yield (5.42±0.88 g L^{-1}) was observed for *Scenedesmus obliquus* in 15 days. The highest lipid accumulation (7.21±0.69 %) was observed for *Chlamydomonas reinhardtii* while *Chlorella sorokiniana* demonstrated accumulation of minimum lipid content (1.21±0.03 %) in 3 days. It also emerged that the highest biomass producer algae, *Scenedesmus obliquus* exhibited lipid accumulation of 5.55±0.47 %.

This study represented an initial investigation into the industrial application of algal oil, with growth conditions designed to mimic natural light cycles. A 14/10 light-dark cycle was selected as it closely resembles natural photoperiods, and the extended dark phase helped in mitigating the photoinhibition caused by prolonged exposure to high light intensity. Additionally, previous studies suggested that a longer dark phase supports recovery and enhances metabolic efficiency, as algae utilize stored carbon reserves, such as starch, during the dark phase to drive lipid biosynthesis. From an industrial perspective, reducing the duration of the light period also aligns with energy efficiency goals by minimizing energy consumption for artificial lighting, making the process more cost-effective.

Major obstacle in lipid extraction always remained the difficulty in disrupting the cell wall leading to the expenses involved in the downstream processing of extraction of lipid from algae. The overall extraction cost of algal lipid accounts for 30-40% of overall bio-oil production cost[15]. Thus, for enhancing lipid recovery efficiency, pretreatment become utmost need to optimise the process. Literature also highlighted the effect of different cell disruption methods (e.g., autoclaving, bead-beating, sonication, osmotic shock, water bath, laser treatment, microwave, and blender) on lipid extraction, showing enhanced lipid recovery[16].

Blending and microwave irradiation are optimal for algal oil extraction due to their simplicity, cost-effectiveness. Also, these methods for cell disruption are compatible with downstream processes like pyrolysis. These methods ensure efficient cell disruption, minimal environmental impact, and preservation of oil quality, making them suitable for both research and industrial applications. Hence, in the present investigation, the use of several cell disruption methods, either alone or in combination, significantly enhanced the recovery of lipids from a variety of algal species. A quick comparison of the impact of different cell disruption strategies is summarized diagrammatically (Fig. 2). The use of a blender alone resulted in a 1.5-fold increase in lipid content ($P<0.05$), while the exclusive use of microwave treatment demonstrated about a 2.5-fold increase in lipid extracts from the algal species used in this study. To maximize output and explore the novelty of the study, a combination of two pretreatment protocols (blender and microwave, termed as combination) was investigated under pre-optimized conditions. This innovation resulted in a nearly 3-fold increase in lipid content, significantly better than the use of the cell disruption techniques alone.

The efficiency of lipid extraction was evaluated and analysed based on lipid content (%) and productivity (mg L^{-1} d^{-1}). Fig. 1 illustrates that the lipid content of algal cultures increased after the log phase of cultivation, peaking on the 6th day, further, showing a significant rise after the 9th day. However, the lipid accumulation rate varied significantly among the algal species investigated. To study this effect, the algal cultures were subjected to a variety of solvents in different extraction protocols to facilitate maximum

recovery of algal oils. For example, using Bligh-Dyer method, lipid content extracted ranged from 8.26±0.23% to 22.12±0.24%, Folch method ranged from 5.56±0.21% to 20.1±0.46%, and the Soxhlet method ranged from 2.22±0.33% to 17.98±0.19%. The Bligh-Dyer method showed the highest impact on lipid extraction for most algal species, due to the significant effect of solvent ratio variations on lipid extraction efficiency[17]. The selection and protocol of solvent utilization were crucial for overall lipid extraction, guided by the principle of "like dissolves like" in chemistry. Organic solvents permeate the cytoplasm through the cell membrane and interact with fatty acids. Non-polar solvents such as chloroform and hexane combine with monounsaturated fatty acids (neutral lipids) via Van der Waals interactions, while polar solvents such as methanol form hydrogen bonds with polyunsaturated and saturated fatty acids (polar lipids), displacing the lipid-protein associations that inhibit lipid extraction. This results in an organic solvent-lipid complex that diffuses across the cell membrane, creating a concentration gradient. Therefore, combining nonpolar and polar organic solvents optimizes neutral lipid extraction: nonpolar solvents dissolve intracellular lipids, and polar solvents disrupt the neutral and polar lipid complex[18]. The Soxhlet method, using only non-polar solvents, results in a lower overall lipid yield compared to the Bligh-Dyer and Folch methods, which use a combination of solvents.

The impact of pretreatment protocols on algal biomass was also demonstrated in this study. The initial pretreatment method involved blending, where cells were disrupted by agitation at ~20,000 rpm, applying shear stress that ruptured the rigid cell walls and facilitated lipid release. The lipid content extracted ranged from 10.09±0.29 % to 35.67±0.55%, with the greatest release was observed in *Chlamydomonas* and *Chlorella* species (2 to 4-fold increases), and the least in *Scenedesmus* species. This is attributed to the differing cell wall structures: *Scenedesmus sp*. has a trilamellar cellulose cell wall, *Chlorella* sp. has a 17-21 nm thick unilamellar cellulose cell wall, and *Chlamydomonas* sp. has a 200 nm thick cellulose-deficient cell wall, making *Scenedesmus sp*. more shear-tolerant, *Chlorella* sp. slightly shear-tolerant, and *Chlamydomonas sp*. shear-sensitive[19].Other pretreatment was microwave method which generated heat and pressure, causing up to 95% cell damage and releasing lipid molecules from the cell matrix. A 2-fold increase in lipid release was reported from *Scenedesmus* sp. using microwave treatment, with 21% lipid extraction under 1000 W of microwave followed by solvent extraction using a chloroform: methanol ratio of 2:1[20]. *Chlorella vulgaris* achieved 18% lipid extraction under 2450 MHz microwave followed by similar solvent extraction (ratio 1:1)[21]. In this study, lipid content ranged from 13.14±0.08% to 41.90±0.66 %, a 2 to 6-fold increase compared to untreated samples in both *Chlamydomonas* and *Chlorella* species. Microwave treatment accelerated solvent partitioning, increasing cell kinetic energy and causing disruption[22]. Larger water volumes are recommended for microwave heating to enhance oil release with polar solvents.

To mitigate excessive heat evolution, a combination of physical disruption and irradiation was used. While combining enzymatic and mechanical cell disruption was considered, its high cost and limitations for large-scale bioreactors made it less suitable[23]. This study's combination of mechanical (blender), physical (microwave), and chemical (solvent) extraction methodologies for algal lipid recovery is novel. This approach reduced heat generation and treatment time, tripling lipid extraction rates. This also highlighted the efficacy of utilization of cell disruption technique by blending and distorting the structure of algal cell membrane by appropriate wavelength microwave technology, followed by extraction of released oil using appropriate solvents e.g., chloroform: methanol etc.

Some reports are available in literature wherein the lipid yield has been reported in the range of ~14 % to 22 %[20,24-25] by the exclusive use of microwave as “the” pretreatment protocol. However, in the present investigation, the microwave treatment protocol was modified by fixation of the radiation time as 5 min with “a pause of few seconds” to limit the rise in temperature to 100°C. This modification in time of microwave heating improved the lipid yield to ~22 % to 37 % for the same algal species. The other method of pretreatment was the use of blender wherein the literature studies reported that the lipid yield of ~20 % to 25 %[26-28] (Table 3). However, in the present investigation, the blender pretreatment method exhibited improved results by introduction of “off time” of 1 min during a 5 min cycle of blending. This alteration led to an improved lipid yield in the range of ~28 % to 35 %.

The selection of pretreatment conditions, such as rpm, time, and temperature, was based on optimizing the balance between effective cell disruption and preserving the integrity of lipids for extraction. For

Table 3 — Yield of algal oil (lipid from) by application of different pretreatment method and lipid extraction protocols

Pretreatment method	Operational Conditions	Algal species	Lipid Yield (Y) (%)	Reference
Microwave	Time: 1 min Chloroform-methanol (2:1)	*Chlamydomonas sp.*	22.6	24
	Temp: 150°C Time: 5 min Chloroform-methanol (1:2)	*Chlorella sorokiniana*	14.2	25
	Temp: 100°C Time: 10 min freeze dried samples Chloroform-methanol (2:1)	*Scenedesmus obliquus*	21	20
	Temp: 100 ˚C Time: 5 min Chloroform-methanol (2:1)	*Chlamydomonas reinhardtii*	37.78	Present Study
	Temp: 100°C Time: 5 min Chloroform-methanol (1:2)	*Chlorella sorokiniana*	22.56	
	Temp: 100°C Time: 5 min Chloroform-methanol (2:1)	*Scenedesmus obliquus*	36.21	
Blender	Time: 20 min Speed: 20,000 rpm Chloroform-methanol (2:1)	*Chlorella sp.*	20	26
	Chloroform-methanol (2:1)	*Chlorella vulgaris*	25	27
	Temp: 35°C Time: 30 min Chloroform-methanol (2:1)	*Scenedesmus sp.*	25	28
	Time: 5 min Speed: 20,000 rpm Chloroform-methanol (2:1)	*Chlorella pyrenoidosa*	29.54	Present study
	Chloroform-methanol (1:2)	*Chlorella pyrenoidosa*	35.67	
Combination (B+M)	Blender ON Time: 1 min Speed: 20,000 rpm Microwave ON MF: 2,450 MHz MR: 800 W Temp: 100°C Time: 2 min B→M→B→M→B→M Chloroform-methanol (1:2)	*Chlamydomonas reinhardtii* *Chlorella pyrenoidosa* *Chlorella sorokiniana* *Chlorella vulgaris* *Scenedesmus abundans* *Scenedesmus obliquus*	52.08 51.28 24.71 25.11 37.58 42.60	Present study
Lipid Extraction Method	Operational Conditions	Algal Species	Lipid Yield (Y) (in %)	References
Bligh & Dyer	Chloroform: Methanol (2:1) Temp: 30–60°C Time: 15–40 min	*Chlorella sp.*	25	29
	Chloroform: Methanol (2:1) Vortexed at 250 rpm	*Chlorella pyrenoidosa*	20	30
	Chloroform-methanol (1:2)	*Chlorella pyrenoidosa*	13.65	Present
	Temp: 30–60°C	*Chlorella sorokiniana*	8.26	study
Folch	Chloroform: Methanol (2:1)	*Chlorella vulgaris*	36	31
	Chloroform-methanol (2:1)	*Chlorella vulgaris*	9.76	Present study

(*Contd*.)

Table 3 — Yield of algal oil (lipid from) by application of different pretreatment method and lipid extraction protocols (*Contd.*)

Pretreatment method	Operational Conditions	Algal species	Lipid Yield (Y) (%)	Reference
Soxhlet	n-Hexane: Methanol (1:1) Temp: 22–26℃ Time: 2 min	*Chlorella vulgaris*	24	32
	Hexane: Isopropanol (3:2) Temp: room temp. Time: 2 h, 300 rpm	*Scenedesmus sp.*	29	33
	n-Hexane Temp: 65℃ Time: 3 h	*Chlorella vulgaris* *Scenedesmus abundans* *Scenedesmus obliquus*	7.21 17.98 10.38	Present study

the blending method, parameters like rpm and time were chosen to maximize mechanical shearing while minimizing overheating, which could degrade sensitive lipid molecules. The optimal blending speed (rpm) was determined through preliminary trials that assessed the extent of cell wall rupture without excessive energy input. In the microwave method, irradiation time and power settings were selected to achieve uniform heating and rapid cell wall rupture. These conditions were optimized based on experiments that evaluated lipid yield and avoided prolonged exposure to prevent lipid degradation. Overall, the pretreatment conditions were fine-tuned to enhance lipid recovery while maintaining process efficiency and energy economy.

To further improve the oil yield from algae, a combination method consisting of "Blender and Microwave exposure" was developed in the present investigation which led to still higher lipid yield which is not reported so far in the literation. This unique method features crushing and radiation of algal samples which features deep mixing for 1 min in Blender followed by microwave treatment to improvise the enhanced efficiency of radiation penetration. This innovative intracellular oil extraction protocol facilitated enhanced release of algal lipid. This combined method yielded the highest lipid content, ranging from 16.84% to 54.3%. *Chlamydomonas reinhardtii* and *Chlorella pyrenoidosa* demonstrated the highest lipid content, 54.3% and 52.48%, respectively.

The proposed pretreatment methodology combining blending and microwave irradiation offers significant potential for industrial-scale bio-oil production. The enhanced lipid extraction efficiency, particularly in species like *Chlamydomonas reinhardtii*, demonstrates its suitability for large-scale operations where maximizing lipid yield is critical. This method aligns well with commercial needs due to scalability where both blending and microwaving can be adapted for continuous processes using industrial-scale equipment. The reduction in chemical usage and improved lipid recovery reduces operational costs, making the process more economically viable. The process is compatible with a range of traditional extraction methods, allowing industries to integrate it into existing workflows with minimal modifications. The ability to increase lipid recovery supports sustainable bio-oil production, aligning with global goals for renewable energy sources.

The proposed approach also has several challenges that need to be addressed before industrial application such as energy requirements. While efficient, both blending and microwaving consume energy, and optimizing these processes for large-scale applications without significant energy trade-offs will be essential. Industrial-scale microwaves and high-capacity blenders may involve substantial upfront costs, impacting initial investment. The efficacy of pretreatment varies across species. Ensuring consistent performance across diverse algal biomass types will require further optimization. Seamlessly integrating this pretreatment into continuous bio-oil production systems while maintaining efficiency and throughput could be challenging. Prolonged or excessive microwave irradiation may risk thermal degradation of sensitive lipid molecules, necessitating precise control over operating parameters. Scaling up requires a reliable and sustainable supply of algal biomass, which depends on advances in algae cultivation and harvesting technologies.

Each pretreatment protocol for extraction of lipid (oil) from algal cells has its own advantages and disadvantages as it emerged from the comparative

analysis. However, no attempt has been made by any investigators to highlight the strategic role of combining different pretreatment protocols and analysing the improvement in efficiency of lipid extraction. The present investigation attempts to fill in this gap in the literature reports to facilitate the possible commercialization of this innovative algal oil recovery technology so that it is available for different chemical process plants and automobile industry to eliminate exclusive dependence on fossil fuels.

Conclusion

Among the pretreatment and lipid extraction methods tested on six algal species, the newly designed 'combination' pretreatment method followed by the Bligh-Dyer extraction technique was the most effective for enhancing lipid recovery. *Chlorella pyrenoidosa* exhibited the highest lipid yield with this approach. The improved extraction methodology increased lipid output by 2 to 6 times compared to standard methods. In terms of lipid content, *Chlamydomonas reinhardtii* and *Chlorella pyrenoidosa* were the top-performing species, producing approximately 54% and 52% lipid content, respectively, as a percentage of their dry biomass. Furthermore, the combination pretreatment method proved effective across various extraction techniques, including Bligh-Dyer, Folch, and Soxhlet. This effectiveness can be attributed to the extensive disruption of the algal cell walls, maximizing lipid release.

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