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Optimization of Lipid Recovery from *Aurantiochytrium limacinum* SR21 Biomass Using Ultrasound-assisted Extraction

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Abstract

Lipid extraction from microalgae is of great interest as a future resource for various industries. However, the current extraction methods frequently involve the use of solvents that can be costly and environmentally harmful. In this study, an ultrasound-assisted extraction technique was proposed to enhance lipid extraction from *Aurantiochytrium limacinum* SR21 biomass. This technique optimizes the processes to eliminate the need for solvent, thereby helping alleviate environmental issues associated with the use of solvents. Under optimal conditions (NaCl concentration, 15% [w/v]; ultrasonic frequency, 45 kHz; temperature, 40 ºC; and treatment time, 30 min), the lipid yield was 27.35%. Further, a comparison of lipid extraction yield under different frequencies at fixed positions within the ultrasonication bath revealed the highest lipid yield (39.52%) under a frequency of 45 kHz, followed by 28 kHz (20.36%) and 100 kHz (13.17%). Therefore, ultrasound-assisted extraction is promising for efficient solvent-free lipid extraction.

Keywords: Microalgae; Lipid extraction; Solvent-free extraction; Ultrasonic bath assisted; Cavitation intensity

1. Introduction

The recent global demand for valuable compounds from microorganisms, such as lipids from microalgae, has become a matter of global importance and promoted the search for efficient and sustainable extraction methods. This demand stems from the versatile applications of these compounds in various industries, including the dietary, cosmetic, and energy sectors. One such remarkable compound with several benefits, directly derived from microalgae, is docosahexaenoic acid (DHA). DHA is an essential omega-3 polyunsaturated fatty acid (PUFA) that plays a crucial role in the maintenance of human health. Specifically, it is known for its numerous benefits, such as its ability to support brain development and function, prevent cardiovascular diseases, and exert anti-inflammatory effects (Li *et al.,* 2021). The growing awareness of the health benefits of DHA has influenced the global demand for its production, which has been steadily increasing (Liu *et al.,* 2023). However, a crucial concern regarding the sustainability and environmental impact of relying on fish as the primary source of DHA worldwide is whether the ever-increasing global demand can be met. Further, traditional fish oil production methods have negative environmental impacts, including ecosystem disruption (Bartek *et al.,* 2021). Therefore, to address concerns regarding

the limited availability of DHA from fish and its associated environmental impact, efficient sources of DHA are being explored (Abu-Ghosh *et al.,* 2021).

Microalgae represent an environmentally responsible alternative that can address the global demand for DHA owing to their ability to accumulate lipids based on genetic modifications (Khoo *et al.,* 2023). Notably, they can also be cultivated during wastewater bioremediation by microalgal-bacterial co-cultivation (Leong *et al.,* 2019b). Thus, these biotechnological developments to promote microalgae lipid synthesis have provided new opportunities for various applications in biotechnology, bioenergy, and other industries that are reliant on microalgae lipid-derived products. Among the various microalgae strains, *Schizochytrium* sp., which is currently classified as *Aurantiochytrium,* has shown particular promise and is known for its ability to accumulate high levels of DHA. Particularly, the strain, *Aurantiochytrium limacinum* SR21, exhibits a high ability to accumulate high levels of DHA content. Previous studies, such as the one conducted by Morita *et al.* (2006), have investigated the relationship between total lipid and DHA, indicating a DHA yield ranging from 32% to 38% of the total lipid from this species, which is comparable to that found in fish oil (Zhu *et al.,* 2007). This makes this species an attractive candidate for further exploration and optimization with respect to DHA production, especially through extraction processes.

To develop such extraction processes, it is necessary to consider prospective technology and the overall global demand. In addition, the process should be associated with efficient resource utilization and consider the impacts on the environment and human health. However, most of the techniques that are commonly used for lipid extraction from *A. limacinum* SR21 biomass, including the modified Folch method (Aida *et al.,* 2017; Leong *et al.,* 2019a), modified Lewis trans-esterification method (Olsen *et al.,* 2023), sonication with a chloroform/methanol solvent mixture (Huang *et al.,* 2012; Nakazawa *et al.,* 2012), bead milling (Byreddy *et al.,* 2016), Kates method with a chloroform/methanol/water (CMW) mixture (Rosa *et al.,* 2010), and CO_2 supercritical fluid extraction (CO_2-SFE) (Delgado Naranjo *et al.,* 2021), still require the use of solvents. The challenge lies in developing an extraction strategy for enhancing lipid recovery from microalgae without the use of solvents. Ultrasound-assisted extraction (UAE) is a promising approach for achieving efficient lipid extraction via cavitation and microstreaming (Khoo *et al.,* 2020; Sivaramakrishnan and Incharoensakdi, 2018). Additionally, it offers several advantages over traditional extraction methods, including shorter extraction times and improved extraction yields. Thus, it can contribute to the development of sustainable and efficient lipid extraction processes with environmental benefits (Carreira-Casais *et al.,* 2021).

This study focused on the use of UAE as an innovative and solvent-free extraction technique and systematically explored various parameters (e.g., ultrasonic frequency, treatment time, temperature, and NaCl concentration) to enhance lipid recovery from *A. limacinum* SR21 biomass This process may enhance the linked DHA yield in the future.

2. Methodology

2.1 Preparation of A. limacinum SR21 biomass

A solution of the wet *A. limacinum* SR21 microalgal sample, which was obtained from AlgaleX Company Limited (Okinawa, Japan), was used in the experiments. The microalgae strain was cultivated under controlled laboratory conditions using a 90-L jar fermenter containing basal medium (per 1 liter; 0.5 L of Awamori distillery waste liquid, 0.5 L of water, 20 g of glucose, and 1.6 g of red sea salt) at 25 ºC for 60 h. The culture conditions for *A. limacinum* SR21 were optimized by AlgaleX Company Limited, and the materials used in this study were prepared using the culture under optimized conditions. Thereafter, microalgae solutions (approximately 2 g) were collected in individual 15-mL polypropylene test tubes with screw caps and stored at –20 °C until further processing.

2.2 Cavitation intensity assessment

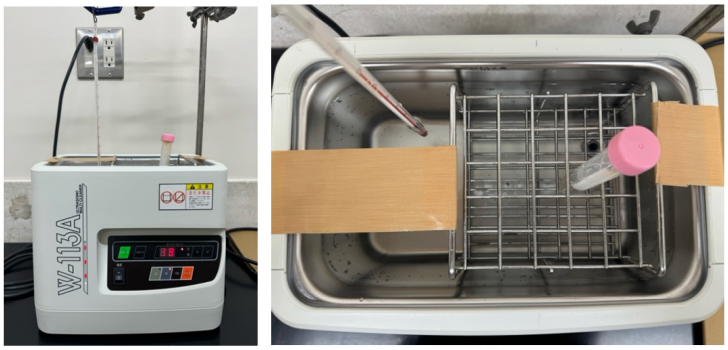
A cavitation intensity assessment was performed to clarify the optimal conditions for enhancing solvent-free lipid extraction. The study initially explored the mapping of cavitation intensity. During this step, experimental studies were conducted using an ultrasonic cleaning cavitation intensity meter (CM-3-100, Alexy Associates Corp., Bethel, NY, USA) at several positions within an ultrasonic device (W-113A, Honda Electronics, Co., Ltd, Japan), which is specifically designed to offer different frequencies (28 kHz, 45 kHz, and 100 kHz) with 100 W as output power. Once the mapping of cavitation was assessed, the effect of different positions within the bath on the extraction process was examined at different frequencies (Figure 1(a)). This mapping led to the identification of cavitation "hotspots" where bubble formation and collapse were the most intense and effective at the different ultrasonic frequencies (Figure 1(b)).

2.3 Ultrasound-assisted extraction

Once the optimal ultrasonic frequency was determined, the optimal treatment time and temperature were also determined. NaCl solution of a suitable concentration was required to enhance overall lipid recovery from the microalgal biomass via the sedimentation of the biomass. This makes it easier to separate the lipid-rich upper layer during the extraction process (Kumar *et al.,* 2015). Therefore, considering NaCl concentration as a parameter in the systematic experimental design (e.g., optimal ultrasonic frequency, treatment time, and treatment temperature) was essential to ensure that the concentration of the NaCl solution used allowed maximum efficiency.

The optimization processes to enhance lipid recovery were investigated under a specific "hotspot" position, which in this case was position no. 15 (as indicated in Figure 1(b)). The use of this position allowed for greater consistency with the results of preliminary experiments. In addition, to explore the optimization processes performed at the hotspot position and investigate the impact of cavitation intensity mapping on lipid yield under optimal conditions, the experiments were conducted by mapping several points covering the entire area of the ultrasonic bath, as shown in Figure 1(b).

Lipid recovery after the completion of each treatment was performed via centrifugation at 2,500 rpm for 10 min to separate the lipid-rich upper layer from the rest of the solution. Next, hexane (3 mL) was added to the upper layer to separate the extracted lipid. Thereafter, this upper layer was collected and evaporated at 40 °C with nitrogen purging. This was followed by measuring the weight of the test tube containing the lipid extract. Total lipid recovery was then calculated using the equation described in Section 2.5. After the final optimal conditions were attained, the results were compared with the control group (no ultrasonication).



(a) (b)

Figure 1. (a) An actual set-up experiment (b) Set-up experiment showing the mapping of a specific cavitation "hotspot" (position no. 15)

2.4 Analysis of total lipid content

The weighed solution of the wet microalgal biomass was collected in a 15-mL polypropylene test tube with a screw cap. Thereafter, total lipid content was determined using a modified Bligh-Dyer and Folch method (Araujo *et al.,* 2011; Bligh and Dyer, 1959; Folch *et al.,* 1956). In brief, chloroform/methanol (2:1) was added to the sample followed by the addition of 0.9% NaCl solution (FUJIILM Wako Pure Chemical Corporation, Osaka, Japan) to the solution layer and vortex mixing for 30 sec. Next, the test tubes were centrifuged at 2,300 rpm for 5 min, and the chloroform layer was collected in a weighed test tube using filter paper (Grade 1 quality, Whatman International Ltd., Maidstone, Kent, UK). Finally, the chloroform was evaporated at 40 °C under nitrogen purging and the weight of the test tube containing the lipid extract was recorded. Total lipid content was then calculated using the following equation:

$$Total\ lipid\ content\ (g\ total\ lipid/g\ wet\ biomass) = \frac{W_a - W_e}{C} \quad (1)$$

where W_a represents the weight of the test tube containing the lipid extract (g), W_e represents the weight of the empty test tube (g), and C represents the weight of the wet biomass sample (g).

The total lipid content of the wet biomass sample was 0.025 g.

2.5 Lipid recovery analysis

For each experimental condition, the following equation was used for lipid content analysis:

$$Lipid\ yield\ (\%\ total\ lipid) = \frac{W_a - W_e}{C \times (Total\ lipid\ content)} \times 100 \quad (2)$$

where W_a represents the weight of the test tube containing the lipid extract (g), W_e represents the weight of the empty test tube (g), and C represents the weight of the wet biomass sample (g).

3. Results and Discussion

3.1 Effect of ultrasonic frequency on lipid recovery

The extraction proceeded sufficiently under different frequencies (28, 45, and 100 kHz) and at several positions within the ultrasonic bath. The results showed that the highest lipid yield was obtained when the experiments were conducted at position 9. This position is strategically located within the ultrasonic bath in a region where the energy distribution and optimal cavitation dynamics are most favorable for efficient lipid release and recovery, leading to the observed highest lipid yield.

Notably, the findings indicated that direct correlations did not occur between the lipid recovery and cavitation intensity level, as shown in the cavitation intensity mapping in Figure 2.

In addition, the lipid yields of the experiments conducted at three different frequencies were significantly different. The lipid yields also appeared to be influenced by the magnitude of the cavitation intensity. Moreover, the dispersion within the ultrasonication bath and the specific locations of the transducers within the bath played a role in this phenomenon. These observations indicated that a frequency of 45 kHz, which corresponded to the highest lipid yield (39.52%; $p < 0.01$), was optimal for lipid recovery, followed by 28 and 100 kHz, which resulted in yields of 20.36% and 13.17% (both $p < 0.01$), respectively. These observations indicated that a moderate frequency was more effective in enhancing lipid extraction. Similarly, Zhang *et al.* (2014) reported that ultrasonication could be used to assist in lipid extraction from oleaginous microorganisms. Low-frequency ultrasonication at 50 Hz resulted in slightly better performance in the extraction process compared to high-frequency ultrasonication at 520 kHz, suggesting that low-frequency ultrasonication was more effective in enhancing the extraction of lipids. This result can be explained by the fact that at low frequencies, cavitation bubbles, which are relatively larger but less numerous, tend to induce physical effects (Mason and Lorimer, 2002; Zhang *et al.,* 2014), such as the mechanical disruption of cell walls or the enhancement of mass transfer through agitation and turbulence. Taken together, these effects contributed to the overall enhancement

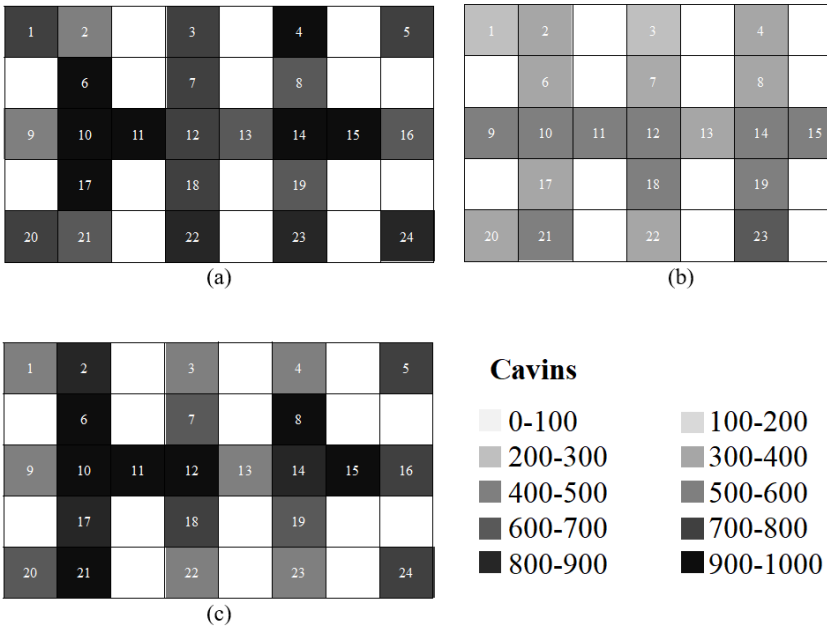


Figure 2. Cavitation intensity measured using the CM-3-100 device at 24 fixed positions within an ultrasonic bath. (a) 28 kHz, (b) 45 kHz, and (c) 100 kHz.

of lipid recovery (Mason *et al.,* 2011, 1996). In contrast, during ultrasonication at a high frequency, the shorter rarefaction time restricted the growth of cavitation bubbles, making it more challenging to reach the threshold for cavitation. This phenomenon at high frequencies can affect the efficiency of certain applications that rely on cavitation effects, such as enhanced mass transfer (Chemat *et al.,* 2017). These results suggest that the optimal frequency for microalgal lipid recovery treatment is in the middle-frequency range of ultrasonication. Moreover, it is important to identify specific hotspots to maximize lipid yield while avoiding over-timing extraction, considering factors such as energy savings, sample preservation, and the broader application of findings for environmental management, especially in the context of environmental emergencies. This integrated approach aligns with the principles of sustainability, resource efficiency, and proactive environmental stewardship.

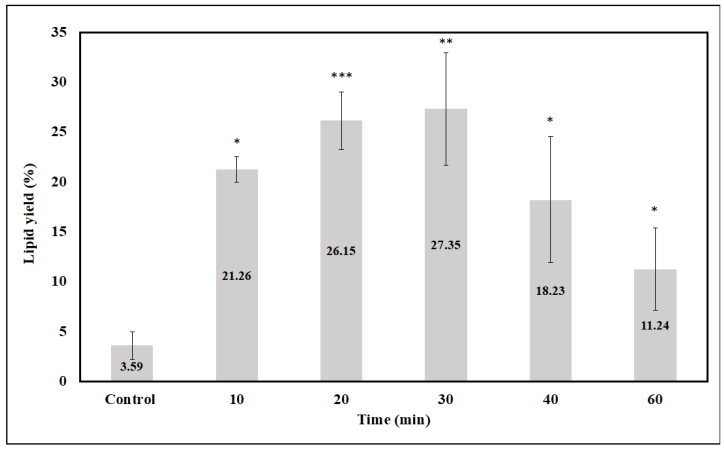
3.2 Effects of ultrasonication times on lipid recovery

Using the identified optimal frequency, the effects of different ultrasonic treatment times (10, 20, 30, 40, and 60 min) on lipid recovery were investigated. The results revealed significant relationships between the ultrasonication time and lipid yield. Increasing the treatment time to 30 min resulted in the highest lipid yield (27.35%). This observation indicated that continuous ultrasonication is a promising strategy for releasing lipids from microalgae cells with rigid cell walls, offering advantages in terms of efficiency and extraction yield (Natarajan *et al.,* 2014). In contrast, extending the treatment time beyond 30 min resulted in a sharp decline in yield rather than improving the lipid output yield (Figure 3(a)). Similarly, Zhang *et al.* (2014) reported that lipid recovery approached its maximum after 20 min of ultrasonication, and the highest lipid recovery of 9.3% was achieved at 30 min. Notably, Park *et al.* (2017) investigated organic solvent-free lipid extraction from wet *Aurantiochytrium* sp. biomass and reported that the highest lipid extraction yield of 77.4% was achieved at a temperature of 150 °C after 30 min of microwave treatment. One plausible explanation for this observation is that the majority of the lipids were released and extracted during the initial stages

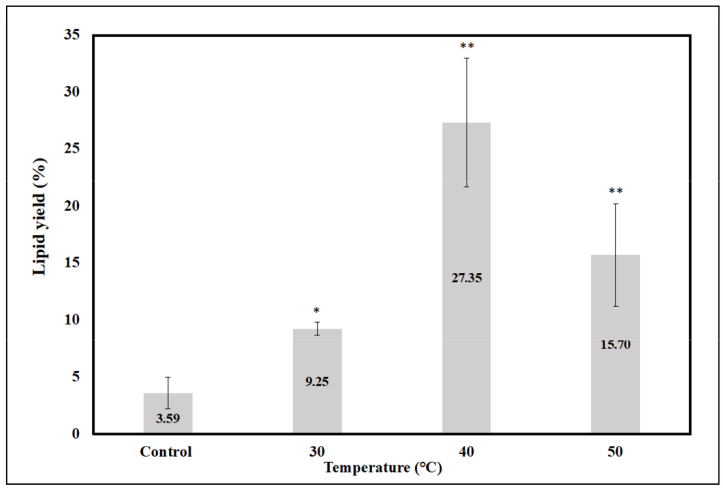
of the extraction process, particularly via the action of bubble cavitation induced by ultrasound irradiation. Lipid recovery facilitated by the bubble collapse mechanism involves several key factors. These factors include cavitation bubble size, intensity, pressure of rarefaction and compression within one cycle, and the energy input required to produce these bubbles (Liu *et al.,* 2022). Together, these actors contribute to the relative efficiency of hydrodynamic cavitation, which is instrumental in improving mass transfer and enhancing lipid recovery from the sample solution (Wu *et al.,* 2019). However, in a previous study, increasing the extraction time did not have any significant effect on lipid yields (Hadiyanto and Adetya, 2018). Therefore, understanding the interplay between viscosity and treatment time is crucial. An increase in viscosity, which is associated with a longer extraction time, can negatively impact lipid recovery. This is because high viscosity may reduce the ability to penetrate the sample matrix, resulting in slower diffusion rates of extracted lipids within the sample (Adam *et al.,* 2012). In the present study, the results revealed a decrease in lipid recovery after treatment times exceeding 30 min, indicating the negative impact of prolonged treatment on lipid extraction efficiency. This decline in lipid recovery may be attributed to the analytical process of centrifugation, which can encounter difficulties in separating viscous lipids from the rest of the solution. Consequently, longer treatment times may lead to lower lipid recovery rates due to the inefficient separation of lipid-containing phases. These observations suggest that a treatment time of 30 min is optimal for lipid recovery in this particular extraction process. Focusing on ultrasonication times, the action of ultrasonication bubbles significantly influences the release and recovery of the majority of lipids. However, prolonging the extraction time might not notably increase lipid yields as longer extraction times can lead to increased viscosity, which could impede lipid recovery and retard the overall extraction process.

3.3 Effects of different temperatures on lipid recovery

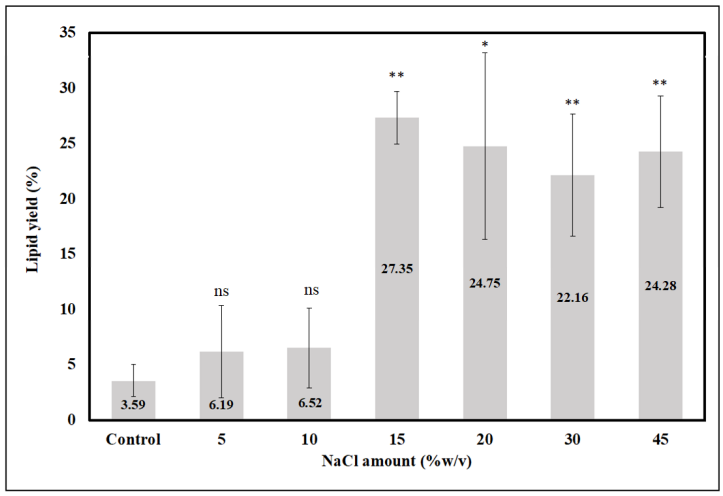
Lipid recovery was investigated under different temperatures (30, 40, and 50 ºC). As shown in Figure 3(b), ultrasonication at 40 °C was optimal for lipid extraction. Beyond 40 °C, no further increases in lipid extraction rate were observed. This result is consistent with the study by Jeevan Kumar and Banerjee (2019) conducted on UAE coupled with binary solvent (chloroform/methanol [2:1, v/v]), which reported that the highest lipid content was obtained at a temperature of 30 °C. The study also reported no significant differences in lipid content when the temperature was increased beyond 40 °C. This observation can be attributed to the decrease in surface tension and increase in vapor pressure that occurs at higher temperatures. As the temperature and surface tension increased and decreased, respectively, it became more difficult for bubbles to form and grow. Therefore, increasing vapor pressure can lead to the premature collapse or instability of cavitation bubbles (King, 2014; Mason and Peters, 2002). The efficiency of ultrasound-assisted processes, including mass transfer and extraction, is influenced by temperature. Although ultrasonic waves can enhance the efficiency of the process by promoting cavitation and improving mass transfer, the effect of sonication may decrease as the temperature increases. An increase in temperature may also influence the stability and lifetime of cavitation bubbles. Higher temperatures can promote bubble growth, destabilize the cavitation process, and lead to bubble collapse. This phenomenon can subsequently result in a decrease in cavitation intensity and less efficient energy transfer to the medium (Mason *et al.,* 1996). Furthermore, thermosonication (TS), a combination of heat and ultrasound, has been shown to enhance the effectiveness of certain processes compared to heat- or ultrasound-only treatments (Abdulstar *et al.,* 2023). However, when the TS involves excessively high temperatures, the benefits of cavitation can be significantly diminished or even negated. This is because high



(a)



(b)



(c)

Figure 3. Effects of different factors on lipid recovery (a) Ultrasonication treatment time, (b) ultrasonication treatment temperature, and (c) NaCl concentration. The control group indicates the experiments conducted without UAE. The asterisks (*) indicate the significance of the data; p-value < 0.05 (*), p < 0.01 (**), and p < 0.001(***).

temperatures negatively affect cavitation activity (Luisa Garcia *et al.,* 1989; Sališová *et al.,* 1997). The effects of different temperatures on lipid recovery reveal that TS enhances various processes. Temperature variations impact surface tension and vapor pressure, affecting bubble formation through cavitation and improving mass transfer which influences lipid recovery. In contrast, the effect of sonication may diminish with increasing temperature leading to a retard in lipid recovery. Therefore, to improve lipid recovery, appropriate temperature is an important factor to be considered.

3.4 Effects of different NaCl concentrations on lipid recovery

Achieving maximum lipid recovery requires a comprehensive approach that considers various factors, including the concentration of NaCl used. The ability of NaCl to assist in layer separation plays a significant role in enhancing lipid recovery from microorganisms. The optimal NaCl concentration is a critical parameter in this process. To determine the optimal NaCl concentration for lipid recovery, NaCl solutions of concentrations 5, 10, 15, 20, 30, and 45% (w/v) were explored in the present study. Figure 3(c), shows that different NaCl concentrations resulted in different lipid yields. The highest yield was obtained at 15% of NaCl, and concentrations below 15% did not significantly enhance lipid yield compared to the control group. Moreover, increases in NaCl concentration above 15% did not have any significant enhancing effect on the lipid yield. Thus, the optimal NaCl concentration for lipid recovery was 15%, possibly providing the optimal osmotic conditions for enhancing lipid extraction from microalgae. The concentration of osmolytes, such as salts (e.g., sodium chloride), in the external solution is a critical factor in determining the efficacy of osmotic shock for lipid extraction. The rapid changes in solute concentration induce mechanical stress on the cell membrane, leading to the degradation of the cell wall in certain organisms and influencing the extent of water movement and cell shrinkage. This approach enables the release of lipid molecules (Russell *et al.,* 2022). Additionally, concentrating protein solutions by NaCl precipitation (salting-out) of proteins from a solution can be achieved by the addition of high concentrations of salts. This increases the ionic strength of the solution and reduces the solubility of proteins, leading them to separate from the solution (Lai *et al.,* 2024). Thus, this phenomenon helps improve the purity of the extracted lipids by reducing contamination from proteins. Specifically, the concentration of NaCl in solution plays a crucial role in modulating ion strength and osmolarity, which in turn influence protein-protein interactions and protein solubility (Roosen-Runge, 2013). Under conditions of optimal salt concentration, the purification or separation of proteins from other cellular components or microalgal component debris is enhanced. Notably, NaCl not only contributes to the precipitation process but also influences the physicochemical properties of the liquid medium (Al-Sabagh *et al.,* 2016), playing an important role in providing optimal osmotic conditions for enhanced lipid extraction. Thus, this innovative approach of improving lipid yield without using solvent presents an interesting approach.

4. Conclusion

In this study, the application of optimal extraction conditions (NaCl concentration 15% [w/v]; ultrasonic frequency, 45 kHz; temperature, 40 ^{o}C; and treatment time, 30 min) resulted in a lipid recovery rate of 27.35%. In addition, in terms of optimal ultrasonic frequencies for extraction, 45 kHz resulted in the highest lipid yield (39.52%), followed by 28 kHz (20.36%), and 100 kHz (13.17%). These observations demonstrated that ultrasonication holds the potential for realizing solvent-free lipid extraction, yet the current processes are limited to extracting extracellular lipids. Future research should focus on achieving solvent-free extraction of both intra- and extracellular lipids from microalgae biomass.

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