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RESEARCH ARTICLE

Fine-tuning of protein extraction from wall-deficient *Chlamydomonas reinhardtii* using liquid nitrogen and sonication-assisted cell disruption

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ABSTRACT

Disruption methods used to extract proteins from the cell often require optimization in terms of yield increase and molecular integrity according to the cell type. Most cell lysis methods primarily target the cell wall. However, even for the wall-deficient strains, efficient extraction of molecules in or attached to membranous structures is a delicate process. In this study, we optimized the protein extraction technique for a cell wall deficient strain of *Chlamydomonas reinhardtii*, which is also a preferred material for most of the recombinant protein production studies. Liquid nitrogen (LN) was evaluated for efficient protein extraction from wall-less strain. The results were compared with sonic treatments, which were optimized in terms of applied power and duration. The results showed that sonication at 25% power for 20 seconds of three rounds provided optimum results for the protein integrity and extraction yield (74.13±2 µg/mL and 185.32±5 mg/g). Although LN has provided similar results in terms of protein content compared to sonication, (70.15±4.43 µg/mL and 175.37±11.09 mg/g maximum), it revealed low efficiency in extracting intact proteins from sub-compartments of the cell.

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Introduction

Algae have been used as a valuable food source for centuries due to their high protein, lipid, and pigment content (Kay, 1991; Wells et al., 2017; Torres-Tiji et al., 2020). Food scarcity caused by climate change in the world has increased the interest in algae as an alternative protein source to plant- and animal-based proteins (Chiong et al., 2016; Bleakley & Hayes, 2017). Microalgae species such as *Chlorella* sp. (Lai et al., 2019) and *Scenedesmus* sp. (Patnaik et al., 2019) are well known natural protein sources utilized as food supplements or feed additives. In addition, microalgae cells have been used effectively to produce recombinant proteins for many years (Gong et al.,

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2011; Rasala & Mayfield, 2015; Doron et al., 2016, Dyo & Purton, 2018). Proteins accumulated by the expression of exogenous genes can be localized in different parts of the cell. Some recombinant products can be stored inside the cytoplasm, while others can accumulate in membranous structures such as chloroplast or mitochondria. Therefore, it is important to consider both the complete lysis of all organelles and the protection of the target molecule for efficient cell disruption.

C. reinhardtii is a widely studied algal species as a model organism at both physiological and molecular levels (Hummel et al., 2012; Saloméa & Merchant, 2019). It is also a host for production of several recombinant proteins such as therapeutics, edible vaccines, or antimicrobials (Rosales-Mendosa et al., 2012; Ahmad et al., 2020). *C. reinhardtii* cell wall consists of hydroxyproline-rich glycoprotein layers (Goodenough & Heuser, 1985) that give robustness to this unicellular organism; however, it also constitutes an obstacle to deliver or extract material of interest.

Cell disruption techniques are widely studied for their cost-effectiveness, energy consumption, and molecular integrity of the target product (D'Hondt et al., 2017; Dixon & Wilken, 2018; Sotto-Sierra et al., 2018). Enzymatic treatment is one of the simple methods used to eliminate the cell wall. *C. reinhardtii* produces its own enzyme called autolysin during the vegetative and sexual stages of its life cycle to lyse the wall structure occurs around zoospores, zygospores and gametes (Jaenicke et al., 1987). However, the use of this enzyme for disruption can result in partial lysis of the cell and may require long incubation periods. In addition, the enzyme can either be bought commercially, which increases the implementation costs, or it can be produced from microalgae itself, which adds an extra step to the disruption process. Cell wall-less species of microalgae such as *Dunaliella salina* is easier to study in terms of cell disruption. Similarly, using a mutant strain without a cell wall is also a preferable strategy providing an advantage for breaking the cell (Lam et al., 2017).

Freeze-thaw is a simple disruption technique for creating pores in the cell wall and membranes during the thawing of the ice crystals that occurred at the freezing step (Dixon & Wilken, 2018). Liquid nitrogen (LN) provides fast freeze of the cells due to extremely low boiling point (-196°C). Sonication, on the other hand, is based on the formation of cavitation bubbles by ultrasound waves and disruption of the cells by mechanical shear stress (Avhad et al., 2014). Sonication stands out as a more powerful technique with moderate energy consumption for breaking the cell wall and internal membranes compared to the freeze-thaw method, however, it is usually preferred to use two techniques together or to use them in combination with other techniques to obtain better results (Gerde et al., 2012; Sotto-Sierra et al., 2018; Stirk et al., 2020). Both methods have the advantage of avoiding the use of chemicals or the need to remove external materials such as beads. It is also possible to apply heat control in sonication to protect degradable molecules such as proteins and lipids. In this study, the LN and sonication methods were compared and optimized for protein isolation from a wall deficient strain of *C. reinhardtii* concerning yield and structural integrity

Materials and Methods

Strain, Media, and Culture Conditions

C. reinhardtii strain cc-3395 cwd arg7-8 mt- (cell wall deficient strain carrying mutation on ARG7 gene encoding argininosuccinate lyase) was ordered from Chlamydomonas Resource Center (chlamycollection.org). The cells were first grown on agar plates containing TAP medium (Harris, 1989) with addition of 100 µg/mL arginine. A loop of cells was scraped from the plate and inoculated in a 100 mL SGII medium (Sager & Granick, 1953) containing 100 µg/mL arginine (SGII-A) in 250 mL flask. This culture was grown for 6 days on a shaker at 100 rpm under 30 µmol photon m−2 s−1 illumination at 25°C and utilized as the stock culture for the inoculation of larger-scale production. The stock culture was washed twice with fresh SGII-A medium and re-suspended in 30 mL of fresh medium before inoculation of 1.55 L final SGII-A culture medium in a 2 L glass bottle. Mixing and aeration was supplied to the culture by an air pump through a 2 µm filter. The culture was grown for 6 days under the same conditions provided for stock culture. The culture was aliquoted into 50 mL falcon tubes and used in disruption experiments (Figure 1).

Cell Density Measurements

Cell counts were performed at the beginning and the end of the cultivation by a Neauber hemocytometer. Dry weight per mL of the culture was identified by filtration of 50 mL of grown culture and drying the biomass at 65°C overnight. The culture was started with 3.2×10^5 cell/mL and ended at 1.19×10^6 cell/mL on the final day.

Sonication-Assisted Disruption

Three replicates of four falcon tubes with 50 mL of the culture (12 falcons in total) were centrifuged at 4000 rpm at

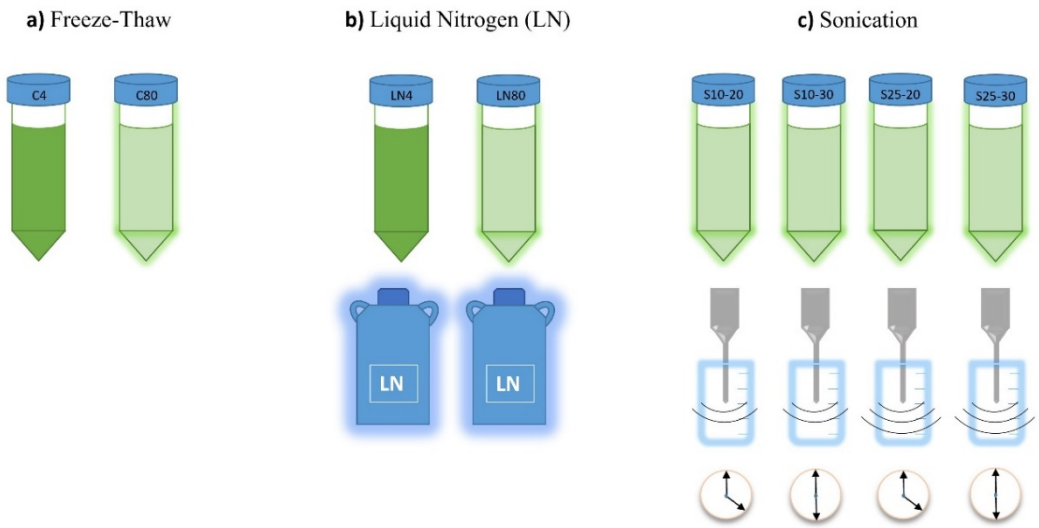

Figure 1. Graphical abstract of the experimental setup

+4°C and cell pellet was kept at -80°C overnight. Frozen cells were partially dissolved on ice and 500 µL of cold TBS containing 0.05% Tween 20 and 1 mM phenylmthylsulfonyl fluoride (PMSF) was added to resuspend and dissolve all the pellet. These cell-solution mixes were transferred into 1.5 mL Eppendorf tubes and kept on ice during the sonication process. Cell disruption was performed by using a 3 mm sonicator probe tip (Bandelin Sonoplus 2070: 70W HF power, 20 kHz) with a 5 cycle, 10% or 25% power, 20 sec or 30 sec durations for 3 complementary rounds (3×) with 10 seconds of resting time in between the rounds for each tube (Table 1). 50 µL of the lysed cells were separated for the later use at microscopic observations. The remaining lysates were centrifuged at 13,400 rpm (max) at +4°C for 25 minutes. Supernatants containing total soluble proteins (TSP) were transferred to new 1.5 mL Eppendorf tubes and kept at +4°C until protein analysis.

Conventional Freeze-Thawing and Use of Liquid Nitrogen

Three replicates of four falcon tubes containing 50 mL of the culture (12 falcons in total) were centrifuged at 4000 rpm at +4°C. Six of the cell pellets were kept at +4°C and remaining 6 of them were frozen at -80°C overnight. All samples were placed on ice and 500 µL of cold TBS containing 0.05% Tween 20 and 1 mM PMSF was added after partial thawing of the -80°C samples. Thawing was completed by continuous pipetting on ice and samples transferred into 1.5 mL Eppendorf tubes. Three replicates from each +4°C and -80°C samples were frozen the second round in liquid nitrogen (LN) for 10 seconds and were partially thawed at room temperature for 6 minutes then placed on ice (Table 1). After completing the thawing process, 50 µL of the lysed cells were separated for microscopic analysis. Remaining lysates were prepared for protein analysis as described for sonication.

Fluorescent microscopy

20 µL of each of the lysed sample was prepared on glass slide for microscopic observations. Chlorophyll autofluorescence was captured at 450 nm excitation spectrum using Leica DM4000B LED fluorescent microscope (Leica, Wetszlar, Germany) and Leica imaging software.

Protein quantification and PAGE analysis

Protein quantifications were measured by Bradford assay (Bradford, 1976). Two standard curves were generated to get more accurate estimations using Bovin Serum Albumin (BSA) as the standard protein molecule; the first one with 10, 20, 30, 40 and 50 µg/mL BSA for smaller amount of protein quantifications as in the control sample (C4) and the second curve with 100, 200, 300, 400 and 500 µg/mL BSA for larger quantities (rest of the samples). 2× Laemmli Buffer with 10% mercaptoethanol was added on to 10 µl of each extract and denatured at 95°C for 5 minutes before gel loading. All 20 µL of each sample was loaded to the gel. PageRuler™ Prestained Protein Ladder 10 to 180 kDa (ThermoFisher Scientific #26616) was loaded to the first lane as protein marker. Proteins were separated on 4-20% polyacrylamide gel (Miniprotean® TGXTM Precast Gels, BioRad, U.S.) at 120 volts and stained in Coomassie Brilliant Blue for 15 minutes and destained overnight on a shaker at 120 rpm.

Table 1. Description of freeze-thaw, liquid nitrogen and sonication methods applied in this study

Freeze-Thaw Control	Parameters
C4	+4°C overnight
C80	-80°C overnight
Liquid Nitrogen	
LN4	C4 + Liquid Nitrogen (-196°C)
LN80	C80 + Liquid Nitrogen (-196°C)
Sonication	
S10-20	C80 + Sonication; 10% power, 20 seconds, 3 rounds
S10-30	C80 + Sonication; 10% power, 30 seconds, 3 rounds
S25-20	C80 + Sonication; 25% power, 20 seconds, 3 rounds
S25-30	C80 + Sonication; 25% power, 30 seconds, 3 rounds

Table 2. Mean values of protein concentrations obtained from each disruption experiment

	µg/mL protein in liquid culture	mg/g protein per dry weight
Freeze-Thaw Control		
C4	0.98+0.24	2.46±0.60
C80	49.11±3.65	122.78±9.12
Liquid Nitrogen		
LN4	62.07±1.72**	155.18±4.29**
LN80	70.15±4.43*	175.37±11.09*
Sonication		
S10-20	59.57±2.95	148.93±7.39
S10-30	60.14±2.42	150.35±6.05
S25-20	74.13±2.00**	185.32±5.00**
S25-30	73.79±5.44**	184.46±13.60**

Note: * indicates significance level is 0.05; ** indicates significance level is 0.01

Statistical analysis

Experiments were conducted as three independent replicates. Statistical significance ($p<0.05$) between groups were determined by two-tailed t-test analysis using Microsoft Excel.

Results and Discussion

Protein concentrations

The results obtained from protein quantification analysis are summarized in Table 2. A considerably higher protein yield was obtained from the cells pre-frozen overnight at -80 degrees (C80) compared to the control culture at +4°C (C4). Liquid nitrogen (LN4) provided significantly higher protein yield compared to both results obtained from C4 and C80 samples ($p<0.005$ and $p<0.05$, respectively) (Figure 2). Remarkably, the results obtained by LN revealed similar amount of protein to the trials performed by sonication.

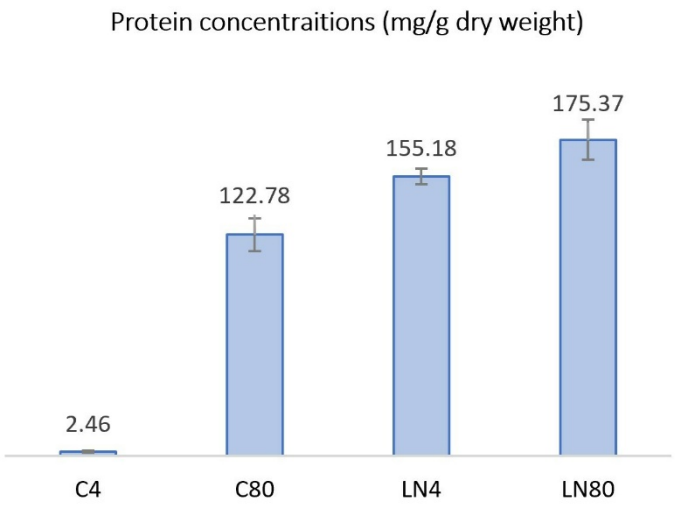

Figure 2. Protein concentrations from the freeze-thaw and LN experiments. (See Table 1 for the codes)

No significant difference for protein concentrations was observed between LN4 and 10% power (S10-20, S10-30) or LN80 and 25% power sonication treatments (S25-20, S25-30),

regardless of the duration. Sonication treatments were evaluated in terms of both the level of power and duration of the application (Figure 3). The applied power rather than the application time emerged as a more important factor for protein recovery. Both 25% power applied samples with 20 and 30 seconds of application times (S25-20, S25-30) provided a higher amount of protein than the samples subjected to disruption with 10% power at the same durations (S10-20, S10-30) ($p<0.05$). Comparing the results of all disruption experiments, sonication for 20 seconds of three rounds using 25% power (S25-20) provided the highest amount of protein in this study (74.13±2 µg/mL and 185.32±5 mg/g).

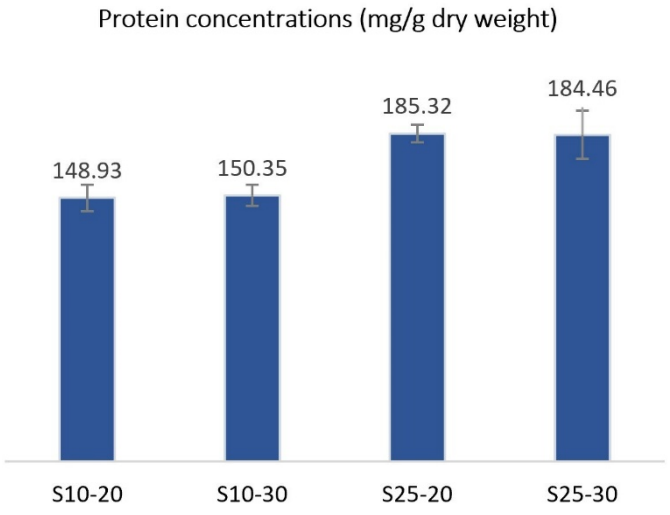

Figure 3. Protein concentrations from the samples sonicated in different application conditions (See Table 1 for the codes)

SDS PAGE analysis

10 µl of each protein extract was loaded on the gel for polyacrylamide gel electrophoresis (PAGE) analysis. The results showed consistency in terms of the concentration of extracted proteins. However, differences were observed in the protein bands obtained from different disruption techniques. The protein bands above 180 kDa (Figure 4, frame 1: *f1*) and around 25 kDa (Figure 4, frame 2: *f2*), provided different results between the application of LN and sonication. A partial degradation was also observed in between the sonication trials (S25-30) for the protein bands in *f1* depending on the duration.

Disruption efficiency

The autofluorescence property of the chlorophylls inside the cell was used to observe the cell lysis efficiency (Figure 5). While the integrity of the cells was preserved in the C4 control, the cells in the C80 samples were visualized as dispersed clusters. Chlorophyll fluorescence could still be observed in the samples that are disrupted with liquid nitrogen. Chlorophyll release was very low in only freeze-thawed samples (data not shown), and autofluorescence was preserved in intracellular structures. On the other hand, very weak autofluorescence signal was obtained from the sonicated samples, which were observed as pale cell residues.

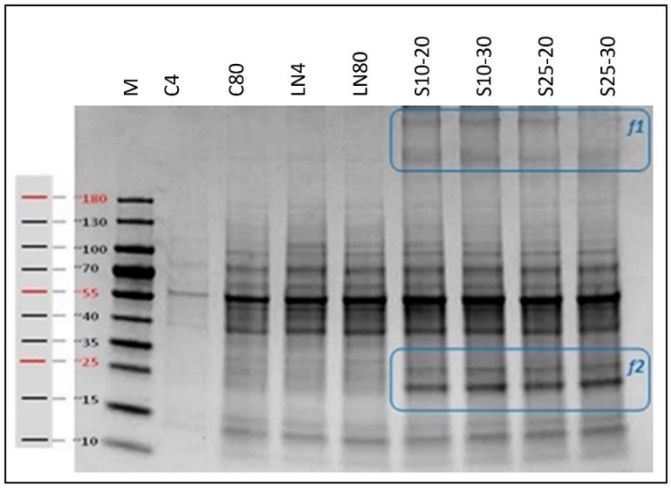

Figure 4. Protein profiles of the disrupted samples obtained from SDS PAGE analysis

Discussion

Proteins constitute approximately 40-60% of microalgae cells (Wang & Yin, 2018). Several techniques have been evaluated as a protein extraction method for *C. reinhardtii* (Newman et al., 1991; Bensalem et al., 2020). Cell wall removal is the main objective in most the cell disruption studies. Extraction of proteins from within the cell begins with cell lysis. Species with a cell wall require more rigorous methods, and the lack of this structure provides an advantage in terms of extraction success. In a previous study, Lam et al. (2017) obtained 3 times higher results with the cell wall-deficient strain of *C. reinhardtii* than the wild type in their study, in which they evaluated the effectiveness of the pulsed electric field (PEF) method in cell disruption. Removing the cell wall with autolysin pre-treatment was also reported to result a significantly higher yield of proteins Sotto-Sierra et al. (2017). Nevertheless, the complete disintegration of the membranous structures in the cell and the separation of proteins without degradation is a sensitive process. In this study, the effects of easy to apply freeze-thaw method, liquid nitrogen use and sonication application, in terms of protein extraction and molecular integrity for a wall-deficient strain of *C. reinhardtii* were compared.

Freeze-thawing technique is usually used in combination with most of the disruption methods. Freezing cells in very low

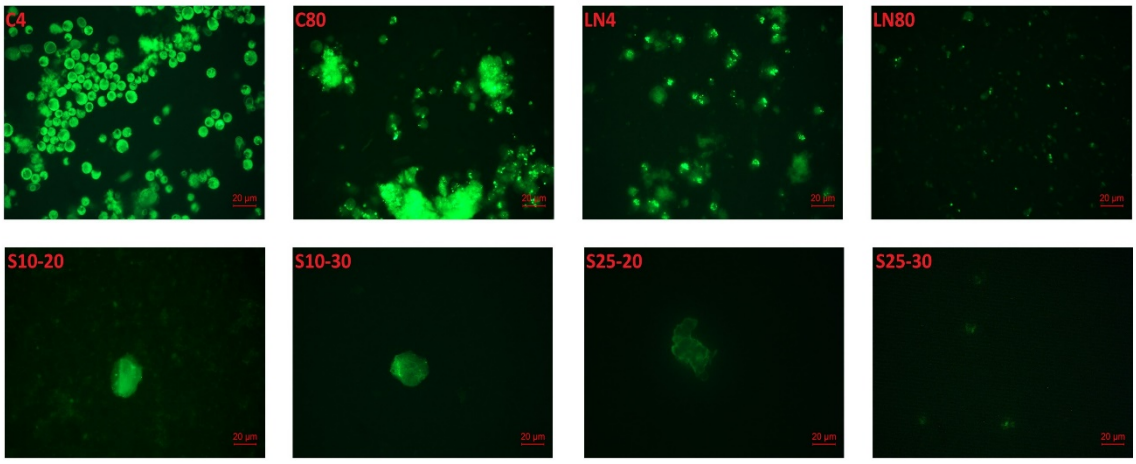

Figure 5. Chlorophyll autofluorescence of each sample after the disruption treatment

temperatures followed by thawing may damage the cell wall in the media due to melting of the ice crystals. However, its effect on the cell membranes is limited. The first step of this study was to investigate the concentration of proteins that can be extracted by keeping the wall-less cells at -80°C overnight (C80).

Disrupting the cells with liquid nitrogen after -80°C freezing step (LN80) resulted the highest amount of protein yield in between the freeze-thawing trials (Figure 2). Quantification analysis using liquid nitrogen revealed similarities with the sonication trials, indicating that freeze-thawing cells with LN would be sufficient to provide proteins for the wall-deficient strain of *C. reinhardtii*.

Sonication is one of the most effective mechanical disruption techniques that can effectively eliminate microalgal cell walls, which may contain cellulose, polysaccharides, and glycoproteins (Kuhavichanan et al., 2018; Alhattab et al., 2019). However, in this study, it was revealed that even in the absence of a cell wall, cell lysis should be planned with care for effective product recovery. Ultrasonic power and processing time are two important parameters for extracting high-value products from microorganisms, including microalgae. (Zheng et al., 2021). Kuhavichanan et al. (2018) reported that increasing the processing time of sonication treatment resulted in higher protein yields for green microalga *Coelastrum* sp. However, in our study, no significant difference was found between the times applied as 20 seconds for 3 rounds (60s in total) and 30 seconds for 3 rounds (90s in total). Instead, higher protein yield was achieved by the increase of applied power (percent amplitude).

Extraction efficiency and molecular integrity was also observed by the SDS PAGE analysis. The weak protein bands observed in C4 were presumably due to partial lysis occurred during centrifugation. The freeze-thaw method alone was insufficient for the extraction of protein bands above 180 kDa (Figure 4, frame 1: *f1*) and around 25 kDa (Figure 4, frame 2: *f2*), even using liquid nitrogen. Protein degradation occurred as the power increased in sonication applications with longer duration (S25-30) for the protein bands in *f1*. 55 kDa and 15 kDa bands of RubisCo protein (Sudhani et al., 2015) were observed in all samples Figure 4. RubisCo is an important protein responsible for carbon fixation in photosynthetic organisms and is localized in the chloroplast stroma in *C. reinhardtii* or in membrane-free mini-organelles called pyrenoids, which are also located within the chloroplast (Borkhsenious et al., 1998). The faint protein bands of about 25 kDa (*f2*) in freeze-thawed samples was presumably due to protein degradation. It is also possible that these bands are coming from subunits or organelles such as mitochondria, which have a small and double-layered membrane structure and therefore are more difficult to lyse with the freeze-thaw method. On the other hand, the protein bands at the same size have been previously reported to be the light-harvesting complex (LHCII) by Sotto-Sierra (2017) referring to White & Melis (2006). LHC proteins are known to be localized in thylakoid membranes of chloroplast in microalgae (Grewe et al., 2014). As a result, sonication turned out to be more effective than freeze-thaw methods, including liquid nitrogen, for complete lysis of the chloroplast.

Chlorophyll autofluorescence is a technique being used to easily detect the photosynthetic efficiency simply by measuring the excess light re-emitted by the chlorophyll molecules during photosynthesis (Maxwell & Johnson 2000). In our study, the autofluorescence of the chlorophylls was used to observe the cell lysis efficiency (Figure 5). Both LN and sonication assisted disruption methods were able to disperse the cell. However, the

weak chlorophyll signals in LN treated samples were presumed to be pigments stuck into the partially disrupted chloroplast. To ensure the efficiency of LN treatment for the wall-deficient strain, further examination in a subcellular level should be conducted.

Conclusion

Microalgae are one of the important sources of natural proteins. Besides their nutritional value, they are also used successfully as a host system for recombinant protein production. It is important to determine the effective method and conditions for the extraction of proteins produced in microalgae. Although wall-deficient strains stand as easier to disintegrate, more elaborate and optimized methods need to be developed for the isolation of sensitive molecules such as proteins. In overall evaluation of this research, considering both the protein concentration and the molecular composition, the most efficient method to extract proteins from the cell wall-deficient *C. reinhardtii* was found to be by the sonication treatment at 25% power for 20 seconds for 3 rounds. Liquid nitrogen use as the second round of freeze-thawing was promising for obtaining high protein yield for this strain. However, using two techniques in combination (freeze-thaw and sonication) resulted in better end products. Since the strain choice of this study is also a well-known and highly preferred material for recombinant protein expression studies, these findings may serve the scientific community who works in that research field as well.

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Compliance With Ethical Standards

Conflict of Interest

The author declares that there is no conflict of interest.

Ethical Approval

For this type of study, formal consent is not required.

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