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# Research Article
# Optimization of Harvesting of Microalgal *Thalassiosira pseudonana* Biomass Using Chitosan Prepared from Shrimp Shell Waste

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## Abstract

**Background and Objective:** The microalaga *Thalassiosira pseudonana* is widely used in aquaculture sector. The harvesting of microalgae biomass from culture medium is the big challenge in terms of technology and economy. The aim of the present study was to evaluate potential use of chitosan prepared from white leg shrimp shells to harvest the biomass of *T. pseudonana* and determine the contents of some biological compounds of the harvested biomass. **Methodology:** Effects of different harvesting conditions (pH, chitosan concentration and flocculation time) on the harvesting efficiencies of the *T. pseudonana* were evaluated. The recovery efficiency of microalgae biomass of chitosan and contents of some biological compounds of the harvested biomass were compared with some metal salts and centrifugation method. The effect of storage temperature on the contents of some biological compounds as well antioxidant properties of the harvested biomass was also examined. **Results:** The suitable conditions for harvesting the biomass were as follows: A pH of 6, chitosan concentration of 4 mg $L^{-1}$ and flocculation time of 10 min. The flocculation efficiency of chitosan was much higher than that of some inorganic compounds including iron chloride ($FeCl_3$), aluminum sulfate ($Al_2(SO_4)_3$) and polyaluminium chloride (PAC). Compared with the centrifugation method, total contents of chlorophyll-a, chlorophyll-b, carotenoid and polyphenol of the microalgal biomass harvested by chitosan was significantly higher. Contents of biological compounds of the biomass significantly decreased after 2 week storage at different temperatures, the lower the storage temperature the higher the stable contents and antioxidant activity. **Conclusion:** Using chitosan as a flocculating agent could be the potential method to harvest the biomass of microalga *T. pseudonana.*

Key words: Biomass recovery, chitosan, microalgae, *Thalassiosira pseudonana*, shrimp shell waste

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Data Availability: All relevant data are within the paper and its supporting information files.

## INTRODUCTION

Microalgae are single-celled organisms that are founded in both seawater and freshwater with different sizes from a couple micrometers to a few hundred micrometers. Microalgae have high nutritional content and are easily digested, suitably used for many aquatic animals. So far, there are more than 40 microalgal species being classified, produced and used as feed for larvae of aquatic animals. Some microalgae, which are cultured and widely used, include *Thalasiossira pseudonana*, *Skeletonema*, *Chaaaetoceros calcitrans*, *Chaetoceros mulleri*, *Nannochloropsis ocular* and *Chlorella minutissima*. Apart from being the feed source for aquatic animals, microalgae are also considered as the potential source of bioactive substances for human health. Microalgal extracts contain several bioactive compounds such as antioxidants (polyphenols, tocopherols, vitamin C, mycosporine-like amino acids) and pigments, such as carotenoids (carotene xanthophyll), chlorophylls and phycobilins (phycocyanin, phycoerythrin), which possess antibacterial, antiviral, antifungal, anti-inflammatory and antitumor properties[1]. Furthermore, microalgae are also considered as the good material source to produce biodiesel, which has the potential to completely replace fossil diesel in the future; biodiesel production yield from microalgae is 10-20 times higher than that from terrestrial plants[2].

In Vietnam, to be used as feed for aquatic animals, microalgae biomass together with culture medium is often supplied directly to pond. This brings the need to transport or pump culture medium containing microalgae biomass to the pond. This will increase production cost and decrease biomass use efficiency. Moreover, in culture medium, there can be some compounds which can negatively affect the health of aquatic animals, especially in the larval stage. To increase efficiency of biofuel production most methods require harvesting biomass, then drying and squeezing/extracting it. Before producing food products or extracting finished products that have biological activity, biomass is also required to be harvested from culture medium. Therefore, to increase efficiency use of microalgae biomass for different purposes, it is needful to recovery them from culture medium.

The harvesting of microalgae biomass from culture medium is the big challenge in terms of technology and economy. Most microalgae have very small sizes from 1-30 μm and low biomass concentration in culture medium from 0.5-2.0 g $L^{-1}$ depending on culture methods. At present, some methods are used to harvest microalgae biomass including gravity sedimentation, filtration, centrifugation and flocculation. The centrifugation method has been used to harvest biomass of several microalgae. However, this method requires a high energy input leading to the increase of production fee. Norsker *et al.*[3] estimated that centrifugation method has output energy fee equivalent to 50% of energy fee in the process of producing biodiesel from microalgae. Furthermore, another disadvantage of centrifugation method is that it can break down cellular structure, resulting in the decrease in nutritional contents of microalgae biomass. Filtration is another common method used to harvest microalgae biomass[4]. However, the most disadvantage of filtration method is that it can only be applied to species with large cell size (>70 μm), such as *Spirulina*[5].

Using flocculating agents is considered as an effective method at reasonable costs[5]. Inorganic reagents such as ferric chloride ($FeCl_3$), aluminum sulfate ($Al_2(SO_4)_3$) and ferric sulfate ($Fe_2(SO_4)_3$) are widely used in microalgae biomass recovery. When being used to harvest microalgae biomass, they will produce large amounts of sludge and change the pH of culture medium, which can kill or prevent the growth of the microalgae and leave a residue in the water. Besides, metal ion salts when existing in microalgae biomass can cause negative impacts on the health of human and animals[6]. Therefore, culture medium in case of reuse must remove these metals.

Chitosan is becoming increasingly important as a natural biopolymer due to its unique combination of properties like biodegradability, biocompatibility, renewability, bioactivity and ecological acceptability. Chitosan is natural organic polyelectrolyte of high molecular weight and high charge density and being widely used in water treatment. It has a net positive charge that allows it to strongly absorb microorganism including negatively charged microalgae[7]. With these features, chitosan is considered as the potential flocculating agent that can be used to harvest microalgae biomass, decreasing fees and increasing quality of harvested microalgae biomass. Vietnam is one of the foremost countries involved in shrimp aquaculture. Shrimp are usually peeled in standard seafood processing factories to obtain shrimp meat for export and the leftover shells and heads, approximately 35-45% of the total weight are considered to be waste. As a result, shrimp processing leads to massive amounts of shrimp biowaste in Vietnam estimated to be more than 200,000 metric tons (wet weight) per year. To date, the shrimp waste in Vietnam has been used primarily for the preparation of chitin and chitosan[8]. In Vietnam, *T. pseudonana* is widely used as live feed for shrimp in the larval stage. At present, before using it as the feed for shrimp, microalgae biomass together with culture medium are pumped directly to pond. This is not cost-effective way to use in a large scale. In addition, some toxic components in the culture medium may

negatively affect animal health as mentioned above. In order to increase the efficiency use of biomass, it is necessary to develop methods to harvest microalgae biomass *T. pseudonana* with low cost and remaining quality of biomass after harvested. So far, the contents of some basic nutrition such as protein and lipid of *T. pseudonana* have been stated, however, data of biological compounds such as polyphenol, chlorophyll, carotenoid and antioxidant properties of this microalgal biomass is still limited. Therefore, the aim of the present study was to evaluate the efficacy of chitosan prepared from white shrimp waste for the harvesting of microalgal species *T. pseudonana* cultured in Vietnam. The contents of some biological compounds of harvested biomass and their stability during storage were also evaluated.

## MATERIALS AND METHODS

**Reagents:** Folin-Ciocalteu reagent and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma-Aldrich (St., Louis, MO). Trichloroacetic acid was purchased from pure chemical industries (Osaka, Japan). Methanol and ethanol of High Performance Liquid Chromatography (HPLC) grade were purchased from MERK company (Darmstadt, Germany). All other chemicals and solvents were analytical grade.

**Microalgal strain cultivation:** *Thalassiosira pseudonana* was kindly provided from the Culture Collection of Algae at Uni-president Aquatic Hatchery Vietnam Co. (Ninh Thuan province, Vietnam). Standard cultures were grown with F/2 medium at 25°C, bubbled with air containing 5% (v/v) $CO_2$ and continuously illuminated with fluorescent lamps (200 μE $m^{-2}$ $sec^{-1}$).

**Chitosan sample preparation:** The waste of white shrimp species (*Penaeus vannamei*) was collected from seafood processing factories in Khanh Hoa province, central Vietnam. The waste contained heads and shells. After sampling, the shrimp waste was transported to the laboratory in iced condition. The production process of chitosan was carried based on a slight modification of a previous approach[8]. The waste was demineralized by soaking in a 4% HCl solution for 24 h with solid/liquid (w/v) ratio of 1/5 at room temperature to get chitin. The partially deproteinized waste was further treated with diluted NaOH at a concentration of 4% (w/v) with treatment durations of 12 h and the solid/liquid ratio (w/v) of 1/5 at 60°C in order to remove the remaining protein. Chitin was then deacetylated by using a 18 M NaOH solution at 70°C for 30 h and room temperature for 72 h to obtain chitosan. The degree of deacetylation (DD) of chitosan was determinate by UV method[9]. Viscosity of chitosan solution was determined with Brookfield viscometer. Measurements were made using a spindle 62 or 63 at 30 rpm in a 1% chitosan solution at 25°C. The viscosity of chitosan solution was reported with centipoise (cP) units. Molecular weight of chitosan was determined by intrinsic viscosity method[10]. The characteristics of chitosan were presented in Table 1.

Table 1: Characteristics of chitosan prepared from the waste of white leg shrimp species (*Penaeus vannamei*)

| Characteristics | Value |
|---|---|
| Color | White |
| Turbidity (FTU) | 23±3 |
| Ash content (%) | 0.65±0.02 |
| Protein content (%) | 0.57±0.08 |
| Viscosity (cps) | 750±61 |
| Molecular weight (Kda) | 541.27±32.80 |
| Degree of deacetylation (%) | 70±5 |

### Flocculation-sedimentation experiments

**Effect of pH and flocculation time:** The effect of pH value on flocculation efficiency was carried out by adjusting the pH of culture medium ranging from pH 4-9 using 1 M sodium hydroxide and 1 N hydrochloric acid using the sedimentation experimental procedure in 250 mL cylinders. In order to obtain homogeneous pH, the medium was mixed rapidly until the required pH value was achieved. After pH tuning, a certain amount of flocculants was added to each beaker. After sedimentation under gravity for different sedimentation times, an aliquot of medium was withdrawn for measuring the optical density at the height of two-thirds from the bottom.

**Effect of different flocculants with different dosages:** Four flocculants (chitosan, PAC, $Al_2(SO_4)_3$ and $FeCl_3$) were used for harvesting *T. pseudonana* from culture medium. All of them were common chemicals that have been proved to be efficient flocculants to many types of microalgae and widely used on many flocculation processes. Several dosages of these flocculants were added to 250 mL culture medium and mixed rapidly for 1 min and then slowly for additional 1 min. Thereafter, an aliquot of medium was taken for measuring the flocculation efficiency at the height of two-thirds from the bottom after sedimentation under gravity.

**Determination of microalgal biomass harvesting efficiencies:** Turbidity of the mixture after flocculation process was measured at 450 nm (optical density at 450 nm) (Carry 50, Varian, Australia). The flocculation efficiency, concentration factor and settleable solid volume fraction were determined

by the method of Sirin *et al.*[11]. The microalga flocculation efficiency was determined according to Eq. 1:

$$\text{Flocculation effciency (\%)} = \left(\frac{B - A}{B}\right) \times 100 \quad (1)$$

where, A is $OD_{450}$ of the culture after flocculation, B is $OD_{450}$ of initial culture

The concentration factor was determined by Eq. 2:

$$\text{Concentration Factor (CF)} = \left(\frac{h0}{hf}\right) \times \text{flocculation efficiency} \quad (2)$$

where, $h_o$ is initial height of examined algae solution and $h_f$ is final height of concentrated algae solution. Concentration Factor (CF) is the ratio of final product concentration to the initial concentration.

The settleable solid volume fraction was determined using Eq. 3:

$$\text{Settleable Solid Volume Fraction (SSVF)} = \frac{hf}{h0} \quad (3)$$

The SSVF is a fraction of the initial volume to be further processed, leading to a lower energy path of harvesting; the lower the SSVF, the better.

**Storage treatments:** The biomass of *T. pseudonana* harvested by chitosan was placed in a 50 mL centrifuge tube. The microalgal biomass was stored at different temperatures (-20 and 4°C and Room Temperature (RT)) in dark conditions. During storage, a certain amount of sample was withdrawn at time intervals of 0, 1 and 2 weeks for analysis.

**Determination of contents of some biological compounds:** Accurately weighted 2 g of fresh microalgal biomass was taken and homogenized in tissue homogenizer with 30 mL of aqueous ethanol solution (95%). Homogenized sample mixture was centrifuge for 10,000 rpm for 10 min at 4°C. The supernatant was collected and used for the determination of the contents of total chlorophyll-a, chlorophyll-b, carotenoids and phenolics. The equation 4-6 used for the quantification of chlorophyll-a, chlorophyll-b and carotenoids were as follows[12]:

$$\text{Ch-a} = 13.36A_{664} - 5.19A_{649} \quad (4)$$

$$\text{Ch-b} = 27.43A_{649} - 8.12A_{664} \quad (5)$$

$$\text{Cx+c} = (1000A_{470} - 2.13\text{Ch-a} - 97.63\text{Ch-b})/209 \quad (6)$$

Where:

A = Absorbance
Ch-a = Chlorophyll-a
Ch-b = Chlorophyll-b
Cx+c = Carotenoids

The total phenolic content of the extract was spectrophotometrically determined at 750 nm using the Folin-Ciocalteu assay according to the method of Singleton *et al.*[13]. The content of phenolic compounds was estimated using a calibration curve obtained from a diluted series of Gallic Acid (GA) ranging between 0 and 250 mg $mL^{-1}$. The results were expressed as milligram GA equivalents per gram dry weight of the microalgal biomass (mg GAE $g^{-1}$ dry weight).

**Determination of antioxidant activities:** DPPH radical scavenging activity was determined spectrophotometrically according to the method of Blois[14]. Briefly, 1.5 mL of 0.1 mM methanolic DPPH solution was mixed with various amounts of the extract and the final volume was made up to 4 mL with distilled water. The solutions were mixed thoroughly and kept at RT in the dark for 30 min. The absorbance of the mixtures was measured at 517 nm against a blank without DPPH. The DPPH radical scavenging activity was calculated using Eq. 7:

$$\text{DPPH radical scavenging activity (\%)} = \left(\frac{A_{control} - A_{sample}}{A_{control}}\right) \times 100 \quad (7)$$

where, $A_{control}$ is the absorbance of a control without the extract and $A_{sample}$ is the absorbance of the sample mixture containing the extract. The Effective Concentration ($EC_{50}$ value) was defined as the amount of extract required to achieve 50% of the free radical scavenging activity.

The total reducing power ability of the extracts was measured by the following method of Oyaizu[15] with a slight modification as described below. The reduction of $Fe^{3+}$ to $Fe^{2+}$ was determined by measuring absorbance of the Perl Prussian blue complex. Different amounts of the extracts were mixed with 0.5 mL of 1% potassium ferricyanide and 1 mL of 0.2 M sodium phosphate buffer (pH 6.6). The mixtures were incubated at 50°C for 20 min and subsequently, 0.5 mL of 10% trichloroacetic acid was added. The mixtures were made up to a final volume of 5 mL with distilled water. Finally, 0.25 mL of 0.1% ferric chloride was added. Distilled water was used as blank. Absorbance of the mixtures was measured at 700 nm.

**Statistical analysis:** Each value is expressed as the Mean±SD (n = 3). The SPSS version 16.0 for windows (SPSS Inc., Chicago, IL) was used for statistical analysis. Differences among groups

were subjected to one-way analysis of variance (ANOVA) followed by Duncan's multiple range test. A value of $p<0.05$ was considered statistically significant.

## RESULTS AND DISCUSSION

**Effect of pH on the recovery efficiency of microalgal biomass:** The pH is the important factor affecting the recovery efficiency of microalgae biomass. In the present study, the effect of induced pH ranging from 4-9 on the flocculation efficiency of *T. pseudonana* biomass was evaluated. To evaluate generally the recovery efficiency of microalgae biomass, Flocculation Efficiency (FE), coefficient of flocculation (CF) and Settleable Solid Volume Fraction (SSVF) were determined.

The result showed that pH had considerable effect on the recovery efficiency of microalgae biomass (Fig. 1). The recovery efficiency increased between pH 4 and 6, then tended to decrease when pH is from 6-9. At pH 4, the Flocculation Efficiency (FE) was 89.99%, the coefficient of flocculation (CF) was 2.00 and the Settleable Solid Volume Fraction (SSVF) was 0.45, whereas these values at pH 6 were 94.72%, 3.12 and 0.30, respectively. For pH above 6, a marked reduction of flocculation efficiency was observed. In particular, at pH 9, flocculation efficiency decreased by three times ($p<0.05$) compared to pH 6, with FE remained only 34.07%, CF was 1.30 and SSVF was 0.26. Therefore, the optimal pH for maximal flocculation using chitosan was 6.

The effect of pH on the recovery efficiency of biomass is due to the structure change of chitosan at different pH values. The influence of pH on chitosan's molecular structure can be due to differences in the protonation of the biopolymer amine groups and variations in the conformation of the macromolecule chain and in the structure of the flocs[7]. The first hypothesis of pH effect states that at alkaline pH values, positive charge tends to disappear and chitosan is able to produce large and dense flocks. At neutral pH, the microalgal cells have the highest negative charge and the flocculation efficiency of chitosan is enhanced due to the electrostatic interaction between chitosan and the microalgal cells. Meanwhile, at acidic pH values, chitosan becomes a more extended chain and therefore produces smaller looser flocs[6]. On the other hand, the second hypothesis states that at the environment of near neutral pH, it decreases the degree of viscosity of chitosan and increase negative charge on the surface of microalgae cell[16]. It helps microalgal cells to connect easily to positive surface charge of chitosan through electrostatic force. Furthermore, chitosan has an isoelectric

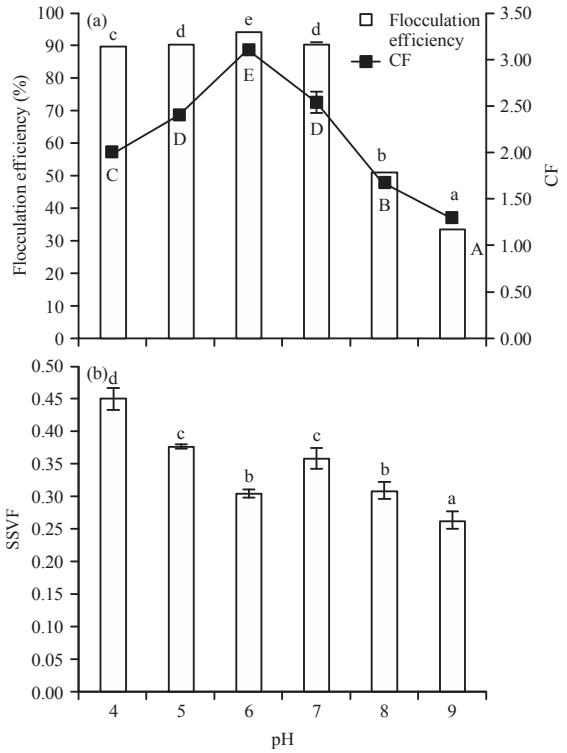


Fig. 1(a-b): Determination of optimal pH for flocculation of the microalga *Thalassiosira pseudonana*, (a) Flocculatin efficiency (FE) and Concentration Factor (CF) and (b) Settleable solid volume fraction (SSVF), Bars with different letters indicate significant differences among treatment groups ($p<0.05$)

point around 6.5 so it has more positive charge at pH 6 than at pH 7 or higher, this will help to increase the recovery efficiency of biomass in the weak acidic environment.

In the present study, the highest recovery efficiency of *T. pseudonana* biomass was obtained at the near neutral pH (pH 6 and 7). These results are appropriate to the second hypothesis mentioned above and in accordance with data from previous studies. According to Xu *et al.*[17], at pH 6, a 100% flocculation efficiency was reached when using chitosan to harvest microalgae biomass *Chlorella sorokiniana*, the recovery efficiency of biomass decreased when pH increased to 7. Similarly, Divakaran and Pillai[18] reported that the recovery efficiency of biomass of *Spirulina*, *Oscillatoria*, *Chlorella* and *Synechocystis* got its highest values when pH was 7. However, some studies stated that the recovery efficiency of microalgae biomass was highest in the alkaline environment. Cheng *et al.*[19] reported that a higher pH at 8.5 was optimal for *Chlorella variabilis*. Similarly, the recovery efficiency of

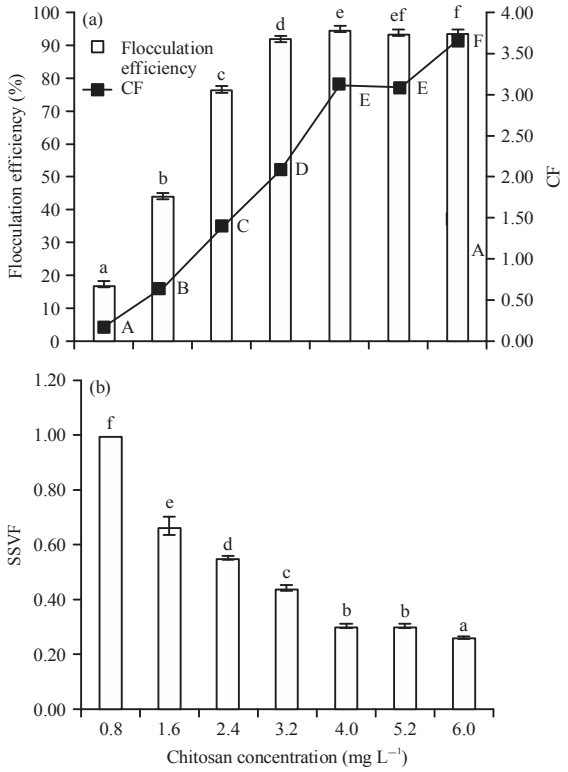


Fig. 2(a-b): Determination of optimal chitosan dosage for flocculation of the microalga *Thalassiosira pseudonana*, (a) Flocculatin efficiency (FE) and Concentration Factor (CF) and (b) Settleable solid volume fraction (SSVF), Bars with different letters indicate significant differences among treatment groups ($p<0.05$)

biomass of *Phaeodactylum tricornutum* by chitosan reached its highest at pH 9.9; over 90% at a chitosan concentration[11] of 20 mg $L^{-1}$. The effect of pH on the recovery efficiency of microalgae biomass depends on culture media, growth conditions and unique strain properties, such as cell morphology, extracellular organic matter and cell surface charge[20]. Therefore, to get the most appropriate pH to harvest microalgae biomass, it is necessary to conduct study for every strain and/or culture medium.

**Effect of chitosan dosages on the recovery efficiency of microalgae biomass:** The concentration of chitosan is also an important factor that affects the recovery efficiency of microalgae biomass. The previous studies showed that to harvest microalgae biomass, the appropriate range of concentration of chitosan from 5-200 mg $L^{-1}$ was needed, depending on microalgal strains[21]. In the present study, the range of chitosan concentration from 0.8-6.0 mg $L^{-1}$ was applied to harvest the biomass of *T. pseudonana*. To conduct this experiment, other parameters including pH and flocculation time were kept fixed.

The result showed that the recovery efficiency of microalgal biomass increased ($p<0.05$) when the concentration of chitosan increased in the range from 0.8-4.0 mg $L^{-1}$ (Fig. 2). However, when the concentration increased up to over 4.0 mg $L^{-1}$, the efficiency obtained was almost maintained ($p\geq0.05$). At the concentration of 0.8 mg $L^{-1}$, the flocculation efficiency was lower 20%, the concentration factor was 0.17 and settleable solid volume fraction was 1.00. The increase of chitosan concentration from 0.8-3.2 mg $L^{-1}$ made the flocculation efficiency being increased by 5 times, the CF factor rose by 12 times and settleable solid volume fraction decreased by 2 times. At the concentration of 4 mg $L^{-1}$, the flocculation efficiency continued to slightly increase. The flocculation efficiency, coefficient of flocculation and settleable solid volume fraction remained hardly unchanged when the concentration of chitosan continued to increase to 6.0 mg $L^{-1}$. Therefore, the appropriate chitosan concentration to harvest biomass was 4.0 mg $L^{-1}$. The trend that increasing concentration of chitosan reduced the recovery efficiency of microalgae biomass could be explained by the phenomenon of charge neutralization and bridging phenomenon. The amino groups of chitosan contain strong positive charge so that they can attract negatively charged microalgae cells, the electrostatic repulsion between microalgae cells drops and the flocculation, therefore is formed. This phenomenon is called as charge neutralization. When the chitosan concentration increases, number of amino group increases and the flocculation ability of microalgal cells are up, too. However, when the chitosan of high concentration is used, such ability can be reduced. The increased number of amino groups creates the repulsion between these groups which makes the flocculation unstable and thereby the efficiency flocculation reduced[22].

The present result was suitable with the previous studies of most of microalgae investigated. Accordingly, the flocculation efficiency of microalgae biomass only increases linearly in the certain concentrations of chitosan. Xu *et al.*[17] reported that at pH 6, the recovery efficiency of the biomass of *Chlorella sorokiniana* gradually increased in accordance with the chitosan concentration from 1-6 mg $mL^{-1}$, however, the recovery efficiency of microalgae biomass started to reduce when the concentration of chitosan continued to increase to 10 mg $mL^{-1}$. Meanwhile, according to Kwon *et al.*[23], the recovery efficiency of microalgae biomass *Tetraselmi* ssp. only gained about 60% when the concentration of chitosan was from 1-3 mg $mL^{-1}$, however, at the level of chitosan concentration of 4.0 mg $mL^{-1}$, the

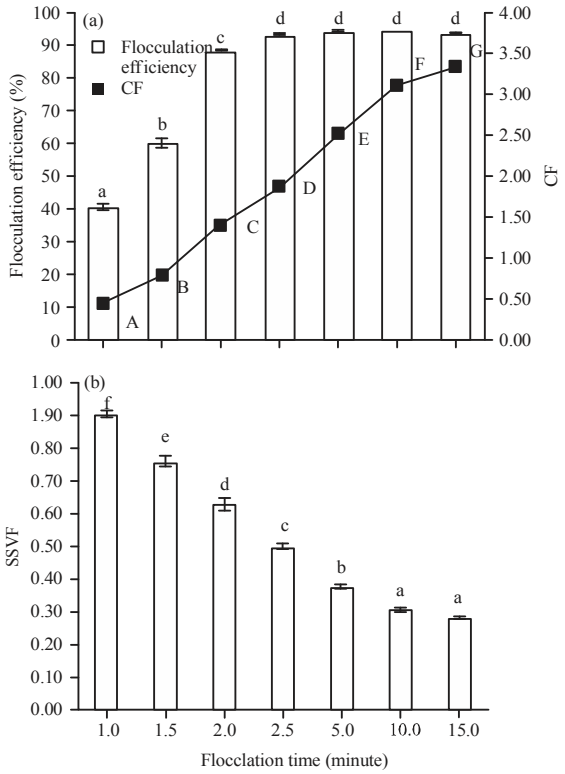


Fig. 3(a-b): Determination of optimal flocculation time for flocculation of the microalga *Thalassiosira pseudonana*, (a) Flocculatin efficiency (FE) and Concentration Factor (CF) and (b) Settleable solid volume fraction (SSVF), Bars with different letters indicate significant differences among treatment groups ($p<0.05$)

recovery reached 80%. The differences in the optimal concentration of chitosan to recover the biomass of microalgae might be due to differences in characteristics of microalgal strains, cell density, culture conditions, pH, temperature and characteristics of chitosan used. The results showed that the chitosan concentration of 4.0 mg $L^{-1}$ at pH of 6 was the most appropriate condition to recover biomass of *T. pseudonana*. This concentration was lower than that of previous studies for other microalgal species. This can be concluded preliminarily that the cost to recover the microalgae *T. pseudonana* will be low.

**Effect of flocculation time on the recovery efficiency of microalgae biomass:** To find the appropriate time for recovering biomass, this experiment was conducted to harvest biomass in the condition of pH and chitosan concentration selected in the previous experiments and at different intervals from 1-15 min. The results were presented in Fig. 3. The results showed that the flocculation time significantly affected the recovery efficiency of microalgal biomass. During the first 2 min and a half, the recovery efficiency of biomass increased rapidly. The flocculation efficiency at 2.5 min increased by 2.3 times compared to the first minute (from 40-93%); SSVF significantly decreased ($p<0.05$), SSVF at 2.5 min felt by 1.8 times compared to the 1st min. During the period of flocculation from 2.5-15 min, the flocculation efficiency of microalgal biomass remained almost unchanged although CF still tended to increase and SSVF tended to decreased. At 5 min of flocculation, CF and SSVF were 2.53 and 0.37, respectively but in 10 min, the CF rose to 3.12 and SSVF felt to 0.30. This proved that the recovery efficiency of microalgal biomass still increased to the flocculation time of 10 min. However, from the 10-15 min of flocculation, the efficiency of flocculation, CF and SSVF remained almost unchanged. This phenomenon may be because the chitosan molecules saturate the number of microalgae cells so that there are no positively charged amino groups to flocculate continuously. Based on the results obtained, a 10-min of flocculation time was chosen for harvesting the biomass of *T. pseudonana*.

**Comparison of the recovery efficiency of microalgae biomass by using chitosan and some inorganic flocculants:** Some metal salts such as $FeCl_3$ and $Al_2(SO4)_3$ and Poly Aluminum Chloride (PAC) are also used in flocculating algal biomass. Therefore, the present study compared the recovery efficiency of the biomass of *T. pseudonana* between chitosan and groups of substances above. Preliminary experiments were conducted to determine the optimum flocculant dosages and pHs. The recovery efficiency of microalgae biomass of this substance group (at appropriate concentrations for each substance) at different pH values was evaluated and compared with chitosan (Table 2). The suitable pH for $FeCl_3$, $Al_2(SO_4)_3$ and PAC were 8, 7 and 5, respectively. At the appropriate pH values, the recovery efficiency of microalgal biomass was 94.71% when chitosan at a concentration of 4 mg $L^{-1}$ was used; this value was equivalent to $FeCl_3$ (87.83%) and $Al_2(SO_4)_3$ (96.52%) at the concentration of 200 mg $L^{-1}$ and PAC at the concentration of 240 mg $L^{-1}$ (Table 2). When assessing the recovery efficiency of microalgal biomass of $FeCl_3$, $Al_2(SO_4)_3$ and PAC at the concentration of 4 mg $L^{-1}$ (the appropriate concentration of chitosan), the recovery efficiency of these substances was almost 0%. Thus, the recovery efficiency of microalgae biomass of chitosan was approximately 50 times higher than that of metal salts and synthetic polymers. The PAC and some heavy metal salts have been used to recover the biomass of some microalgae. The appropriate dosage, pH and flocculation time for

Table 2: Comparison of the flocculation efficiency of chitosan and some inorganic flocculants $FeCl_3$, $Al_2(SO_4)_3$ and PAC at different pH values

| | Flocculation efficiency (%) | | | |
|---|---|---|---|---|
| pH | Chitosan (4 mg $L^{-1}$) | $FeCl_3$ (200 mg $L^{-1}$) | $Al_2(SO_4)_3$ (200 mg $L^{-1}$) | PAC (240 mg $L^{-1}$) |
| 4 | 90.01±0.06 | 15.54±0.01 | 18.41±0.54 | 86.34±0.05 |
| 5 | 90.77±0.09 | 81.65±0.12 | 18.41±0.54 | 91.10±0.16 |
| 6 | 94.71±0.03 | 83.05±0.00 | 88.44±1.01 | 78.00±1.21 |
| 7 | 90.80±0.21 | 68.84±0.00 | 96.52±0.11 | 77.98±0.57 |
| 8 | 51.60± 0.18 | 87.83±0.14 | 92.10±0.11 | 64.70±0.47 |
| 9 | 34.34±0.58 | 79.41±0.00 | 95.61±0.22 | 64.72±0.18 |

these substances also depend on microalgal species. According to Sirin *et al.*[24], the suitable concentration of chitosan (<30 mg $L^{-1}$) to recover the two microalgae *Phaeodactylum tricornutum* and *Nannochloropsis gaditana* was lower than the PAC, $Al_2(SO_4)_3$ and $FeCl_3$ (from 30-70 mg $L^{-1}$). The similar results were also reported by Chen *et al.*[25], the appropriate concentration for microalgae biomass recovery *Scenedesmus* sp. of $FeCl_3$ and $Al_2(SO4)_3$ were 200 and 300 mg $L^{-1}$, respectively; whereas this value of chitosan was only 80 mg $L^{-1}$. These results were consistent with present study. However, some studies indicated that PAC, some heavy metal salts and synthetic organic substances have the suitable concentration lower than that of chitosan to collect some species of microalgae, especially marine microalgae. According to Sirin *et al.*[26], at the appropriate pH, the recovery efficiency of microalgae biomass *Chaetoceros calcitrans* reached about 80% when $Al_2(SO_4)_3$ and PAC were used at the corresponding concentration of 10 and 20 mg $L^{-1}$. Meanwhile, according to the results of Heasman *et al.*[27], up to 80 mg $L^{-1}$ of chitosan was needed to collect 80% of these microalgae biomass.

Although, aluminum sulphate was used as the deposition substances of microalgae and the food in aquaculture, the inorganic precipitation might bring using subjects toxin. The inorganic precipitation could also have negative impacts on the viability of microalgae as well as prevent the ability to recycle and reuse[28]. Although, alum and other inorganic precipitations were relatively cheap compared with some of the synthesis organic flocculating agents but the dosage had to be used much more than organic substances, thus, the cost might be more expensive than using the organic precipitants[29]. When the metal salts with the concentration that is much higher than that of chitosan are used, the content of heavy metals will agglomerate in microalgae biomass, causing negative effect on using subjects. In this study, chitosan provides the recovery efficiency of microalgae biomass *T. pseudonana* that is much higher than that of heavy metal salts and PAC. Therefore, using chitosan to recover biomass of this species not only was highly effective but also had good quality.

**Comparison of the content of some bioactive substances and antioxidant properties of microalgae biomass recovered by chitosan and centrifugation method:** In addition to the method of using flocculants, centrifugation method was also commonly used to harvest many species of microalgae. Although, the centrifugation method is appropriate to recover many species of microalgae with different sizes and culture medium, the most disadvantage of this method is the high cost and to break down the structure of cells, which reduces the quality of the biomass harvested. Therefore, the present study compared the contents of various bioactive compounds including chlorophyll a, chlorophyll-b and total polyphenols and carotenoids as well as antioxidant properties of *T. pseudonana* biomass harvested by chitosan and centrifugation method. Centrifugation of the *T. pseudonana* culture was carried out in 50 mL centrifuge tube with screw caps at 8000 rpm for 10 min at 10°C. The results showed that the content of the studied substances of biomass recovered by chitosan was significantly ($p<0.05$) higher than that of the centrifugation method. The total polyphenol contents of biomass harvested by chitosan and centrifugation method were 7.53 and 4.53 mg GAE $g^{-1}$ dry weight, respectively; the contents of chlorophyll-a, chlorophyll-b and total carotenoid obtained by chitosan were 8.10, 39.63 and 133.29 μg $g^{-1}$ dry weight, respectively; meanwhile this value of the biomass gained by centrifugal method were 7.29; 31.68; 114.31 μg $g^{-1}$ dry weight, respectively (Table 3). Therefore, the microalgae biomass *T. pseudonana* collected by chitosan could maintain the content of bioactive substances better than centrifugation method. The microalgae cell structure collected by chitosan and centrifugation methods was observed in the microscopic pictures (Fig. 4). These pictures showed the differences in the shape and structure of cells. When microalgae biomass was obtained by centrifugation method, the cell walls was broken, initial shapes of cells was lost, whereas cells collected by chitosan and pigment cells could be remained. This explained the loss of content of some substances in the biomass when collected by centrifugation method.

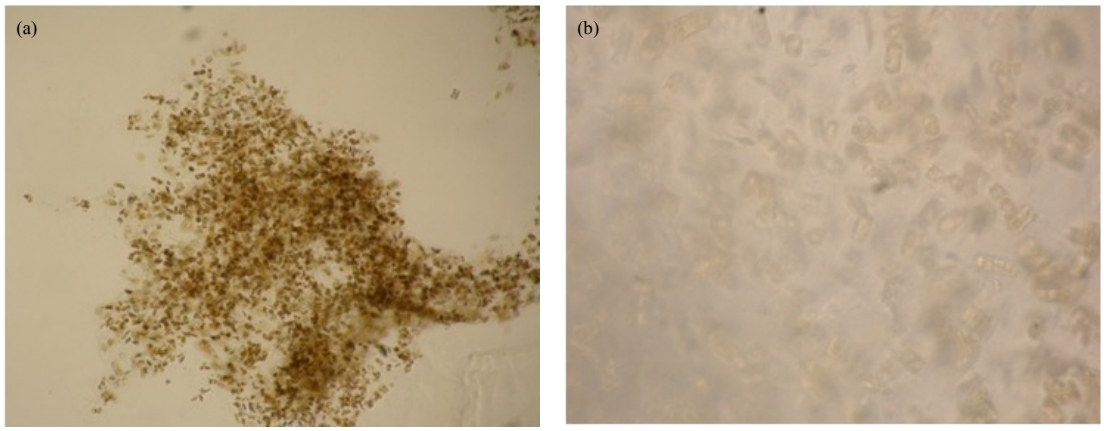


Fig. 4(a-b): Microscopic pictures of microalgal cells harevested by by (a) Chitosan and (b) Centrifugation method

Table 3: Comparison of chlorophyll-a, chlorophyll-b, total carotenoid and total phenolic contents and antioxidant properties of microagal biomass harvested by chitosan and centrifugation method

| Biological compounds | Centrifugation method | Chitosan method |
|---|---|---|
| Chlorophyli- a (μg $g^{-1}$ dry weight) | 7.300±0.44[b] | 8.100±0.91[a] |
| Chlorophyll-b (μg $g^{-1}$ dry weight) | 31.680±1.20[b] | 39.630±4.63[a] |
| Total carotenoid (μg $g^{-1}$ dry weight) | 114.310±1.30[b] | 133.290±3.59[a] |
| Total polyphenol (mg GAE $g^{-1}$ dry weight) | 4.730±0.11[b] | 7.530±0.07[a] |
| DPPH radical scavenging activity ($EC_{50}$, mg $mL^{-1}$) | 0.084±0.002[b] | 0.077±0.001[a] |
| Total reducing power ($EC_{50}$, mg $mL^{-1}$) | 0.304±0.002[b] | 0.294±0.004[a] |

Means with different superscript letters in the same column indicate significant differences ($p<0.05$)

So far, the studies which compared levels of content of some bioactive compounds in microalgae biomass collected by chitosan and centrifugation methods are very limited. The previous studies often focused on determining the content of these compounds in the biomass that was only obtained by a certain method. Hemalatha *et al*.[30] studied to identify the total polyphenol content of three types of microalgae *Navicula clavata*, *Chlorella marina* and *Dunaliella salina* with three different extraction solvents. *Chlorella marina* biomass extracted by methanol has the highest total polyphenol content (0.78 mg GAE $g^{-1}$ dry weight). This result showed that the total polyphenol content in the microalgae *T. pseudonana* is 7.3 times higher than that of *Chlorella marina*. Goiris *et al*.[31] reported the polyphenol content of some microalgal species. Accordingly, the polyphenol content of *Phaeodactylum tricornutum*, *Tetraselmis suecica* and *Nannochloropsis* sp. were 3.75, 1.71 and 1.39 mg GAE $g^{-1}$ dry weight, respectively 1.51, 3.32 and about 4 times lower than the polyphenol content of *T. pseudonana.* Another study of Khuantrairong and Traichaiyaporn[32] on *Cladophora* sp. showed the concentration of chlorophyll-a, chlorophyll-b and total carotenoid were 148.56 and 889 μg $g^{-1}$ dry weight, respectively. It can be see that the content of chlorophyll-a, chlorophyll-b, total carotenoid in microalgae *T. pseudonana* were lower than that of some other species of algae; however, the total polyphenol content was significantly higher. This difference may be because of differences in characteristics of species and culture conditions as well as growing medium.

Microalgae biomass is considered as the nutritious food source. In addition, it also has many important biological activities such as antioxidant, antibacterial, anti-inflammatory capacity and inhibition of cancer cell growth. In the present study, the antioxidant capacity of microalgae biomass *T. pseudonana* collected by chitosan was also evaluated and compared with the centrifugation method. The antioxidant activity was assessed through the ability of scavenging DPPH free radicals and total reducing capacity. The results showed the antioxidant capacity of microalgae biomass collected by chitosan was higher ($p<0.05$) than that of the centrifugation method (Table 3). The $EC_{50}$ values of microalgae biomass recovered by chitosan and centrifugation method when evaluated by DPPH free radical scavenging method were 0.077 and 0.084 mg $mL^{-1}$, respectively; assessed by the total reducing capacity method were 0.294 and 0.304 mg $mL^{-1}$, respectively. These results may be because the centrifugation method had lost a significant amount of substances that have antioxidant capability in the microalgae biomass (Table 3). Some previous studies have shown that chlorophyll-a,

chlorophyll-b, polyphenols and carotenoids were important antioxidants in most of biomass of microalgae.

The antioxidant activities of many different species of microalgae biomass have been evaluated. According to the results of Simic *et al.*[33], the total reducing capacity of green algae *Trentepohlia umbrina* at the concentration of 1 mg $mL^{-1}$ had the absorbance at 700 nm of 0.06; meanwhile at the concentration of 0.89 mg $mL^{-1}$, total reducing capacity total of biomass *T. pseudonan* was ~1.00. According to the result of Shanab *et al.*[34], the ability to reduce DPPH free radical of some microalgae *Nostoc muscorum, Chlorella vulgaris*, *Anabaena flousaquae* and *Phormedium fragile* at the concentration of 50 mg $mL^{-1}$ respectively were 70, 68, 72 and 26 %; whereas at the concentration of 0.14 mg $mL^{-1}$, the ability to reduce DPPH free radical of *T. pseudonana* biomass was up to 59%. The $EC_{50}$ value of algae *Gracilaria changii* evaluated by DPPH free radical method[35] was 14.7 mg $mL^{-1}$ and of *Trentepohlia umbrina*[33] was 0.67 mg $mL^{-1}$. This study showed the $EC_{50}$ value of *T. pseudonana* was lower than that of most of microalgae studied so far, so it can be said that this type of microalgae had strong antioxidant capability. This result was an important basis to confirm the functionality of the microalgae biomass *T. pseudonana* as the nutritious food and biological activities that was a good source for animals.

The ability to revive of microalgal cells recovered by centrifugation and chitosan methods were also investigated. The results showed the ability of microalgal cells to revive after harvested by centrifugation method was only 31% but over 80% was obtained by chitosan method. The difference in the content of bioactive substances, antioxidant capacity and the ability to revive of cells is because the broken cell cannot be rebound when microalgae biomass is recovered by centrifugation method; during the centrifugation process, a number of bioactive substances may also be released from the cell.

**Effects of temperature and storage time on the content of some biological compounds and antioxidant properties:** To have basis information for the storage of microalgae biomass after collecting and using for different purposes, the present study evaluated the effect of storage temperature (-20 and 4°C and RT) on the contents of some bioactive compounds as well antioxidant properties of the harvested biomass. The contents of total chlorophyll-a, chlorophyll-b, carotenoids and polyphenols tended to decrease during storage time. However, the level of change was different according to the storage temperature; the low storage temperature could maintain bioactive substances better (Fig. 5). For example, the content of chlorophyll-a in the first day was 8.096 μg $g^{-1}$ dry weight; this content after 7 days of storage at -20 and 4°C and RT were 7.831, 6.055 and 3.587 g μg $g^{-1}$ dry weight, respectively. The content of chlorophyl a continued to decrease to 6.635, 4.399 and 3.153 μg $g^{-1}$ dry weight, respectively after 14 days of storage at -20 and 4°C and RT. The same trends were observed for total phenolic and carotenoid contents. For example, after 7 days of storage, the total polyphenol contents of microalgae biomass stored at -20 and 4°C and RT were 6.110, 3.180 and 0.690 mg GAE $g^{-1}$ dry weight, respectively. The contents of chlorophyl a, chlorophyl b, total carotenoid and polyphenol felt during storage time because these compounds have strong antioxidant capabilities, they tend to protect other components which are susceptible to oxidation in microalgae biomass and decrease the content. The antioxidant activity of microalgae biomass that changes in storage process was similar to the trend of antioxidant activities (Table 4). The DDPH radical scavenging activity (as evaluated by $EC_{50}$ value) of the microalgal biomass that was stored at -20 and 4°C and RT after 7 days were 0.079, 0.083 and 0.105 mg $mL^{-1}$, respectively. For the total reducing capacity, the $EC_{50}$ value of microalgal biomass during the storage time and temperatures above were 0.305, 0.318 and 0.326 mg $mL^{-1}$, respectively.

To evaluate the quality of the microalgae biomass, the ability of microalgal cells to revive was also evaluated. The density of microalgal cells before storage is 1001667 cells $mL^{-1}$, after 7 days stored at at -20 and 4°C and RT, the cell density are 533333, 366667 and 200000 cells $mL^{-1}$, respectively. According to Harith *et al.*[36], the microalgae biomass *Chaetoceros calsitrans* obtained by chitosan and stored at 4°C gave better results than stored at -20 and 27°C in light and dark conditions. These results were not similar to the present study, this could be explained by differences in characteristics of the microalgal cells. According to Heasman *et al.*[37], storing microalgae biomass at low temperatures could also maintain cell viability. When reducing the storage temperature, the cell's metabolism process, activities of oxidants and vitamins in cells could also be slowed as a result, quality of microalgae might be extended during preservation. From the results obtained, it can initially be concluded that to maintain the quality after recovered by chitosan, biomass should be stored at low temperature. However, the further studies are still needed to investigate to discover the most suitable range of temperatures and storage times.

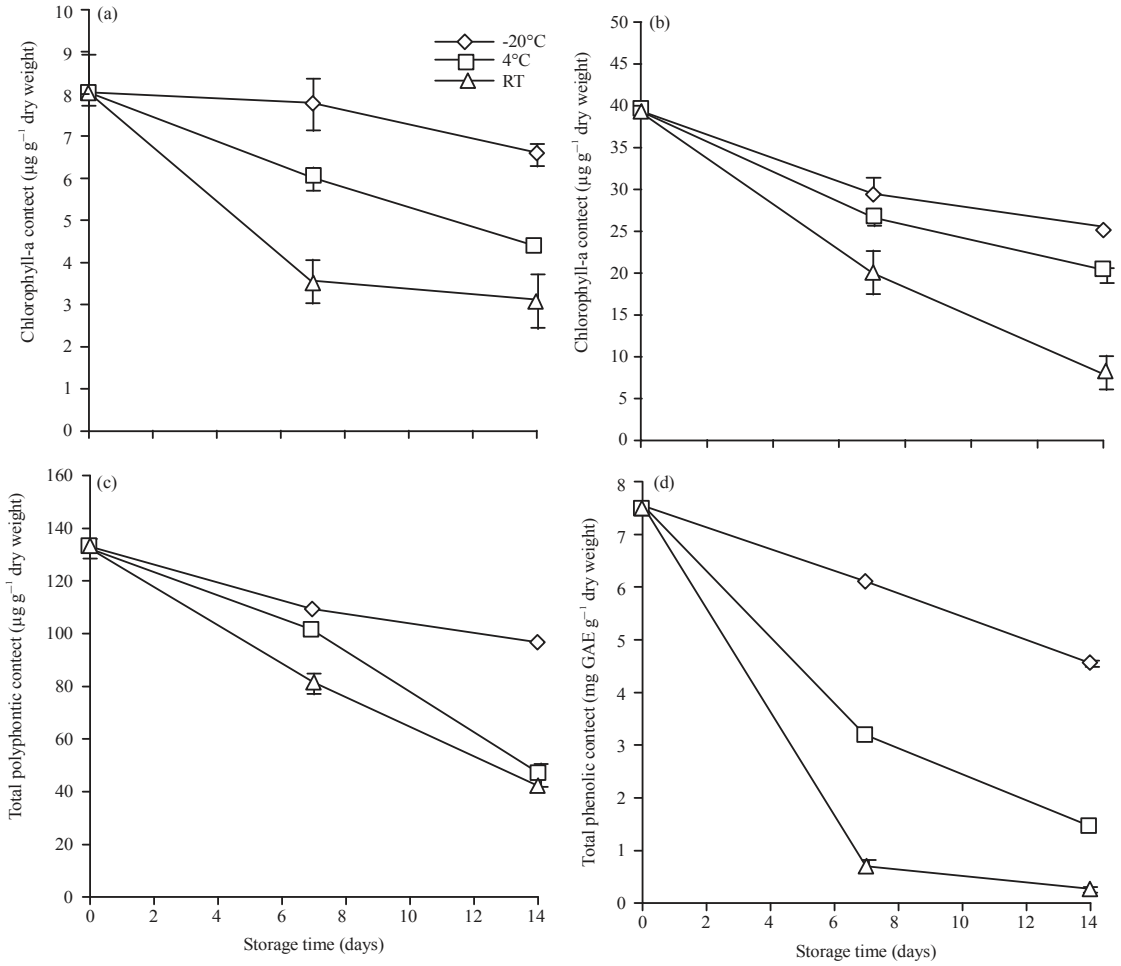


Fig. 5(a-d): Changes in the contents of, (a) Chlorophyll a, (b) Chlorophyll b, (c) Total carotenoid and (d) Total phenolic of microalgal biomass during 14 days of storage under different temperatures

Table 4: Changes in antioxidant activities of microalgal biomass during 14 days of storage under different temperatures

| | DPPH radical scavenging activity ($EC_{50}$, mg $mL^{-1}$) | | | Total reducing power ($EC_{50}$, mg $mL^{-1}$) | | |
|---|---|---|---|---|---|---|
| Storage time (days) | -20°C | 4°C | RT | -20°C | 4°C | RT |
| 0 | 0.077±0.001 | 0.294±0.004 | | | | |
| 7 | 0.079±0.000 | 0.083±0.000 | 0.105±0.001 | 0.305±0.000 | 0.318±0.002 | 0.326±0.002 |
| 14 | 0.089±0.000 | 0.092±0.003 | 0.114±0.002 | 0.320±0.001 | 0.333±0.001 | 0.335±0.005 |

## CONCLUSION

It could be concluded that using chitosan prepared from the waste of white shrimp was both cost-effective and efficient in flocculating of microalga *T. pseudonana* with the efficacy being influenced by induced pH, chitosan dosage and flocculation time. At the optimum condition, nearly 100% of the microalgal biomass was harvested with about 4 mg $L^{-1}$ of chitosan. Thus, chitosan could be a promising flocculant for harvesting the biomass of *T. pseudonana* for aquaculture and any other application. The effects of chitosan characteristics on the harvesting efficiencies of different microalgae and optimization of storage condition for harvested biomass should be conducted in further studies.

## SIGNIFICANCE STATEMENTS

- Chitosan is a highly effective flocculant to harvest *Thalassiosira pseudonana* biomass
- The recovery efficiency of chitosan is much higher than some flocculants

- Using chitosan could maintain bioactive compounds of biomass better than centrifugation method
- Storing the harvested biomass at low temperatures could maintain bioactive compounds

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