Extraction and Quantification of Chlorophyll from Microalgae Chlorella sp.

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Abstract. Algal biomass emerged as a potential source of bioenergy and valuable derivatives in recent years. The major characteristics of microalgae such as high oil contents, carbon sequestration, high growth rate and valuable by-products are leading factors to compete with traditional resources. The aim of current study was (i) to extract and optimize chlorophylls (a and b) at temperature (30-40ºC) and time (60-120 min) by ultrasonication assisted with methanol: hexane (2:1v/v) (ii) to find suitable dilution factor for chlorophyll analysis by UV spectroscopy considering 1:10, 1:15, 1:20 ml/ml and (iii) to determine suitability of dissolving methanol/hexane extract in different solvents for analysis and quantification by simultaneous equations. The full factorial design RSM was employed and found that model is well fitted (0.99 $R^2$). Maximum recovery of total chlorophylls (a and b) was 17.15 µg/ml achieved at 30 ºC and 120 min. The absorbance spectra peaks were found good with a dilution factor of 1:20 ml/ml. Dissolving the extract for analysis in same solvent is suitable choice even though acetone shows sharp peaks, but not in agreement with beer law. These pigments have a high market in pharmaceutical, dietary products, and food industry and recovery of these compounds can play an important role to make bioprocess industry more economical.

Keywords. Quantification, chlorophyll, Microalgae Chlorella sp.

1. Introduction
Microalgae are considered as 3rd generation resource for energy and received high focus in recent years. A microalga is unicellular organism, which possess the tendency to convert sun energy into chemical energy efficiently via photosynthetic process. A variety of strains available in algal class and Chlorella is one of them. Microalgae Chlorella sp. is single celled and green species with 2-10 µm [1]. It contains about 20, 45, 20 % fat, protein, carbohydrate [2,3], and 0.5-1 % pigments per weight of dry biomass [4]. The composition of biomass may vary due to different cultivation media and associated factors. Chlorella has oil contents of 28-32% [5] and abundantly available pigments due to photosynthetic action [6].

Chlorophyll is bioactive compound, which have wide application in pharmaceuticals, food and color industry [7]. Mainly there are two types of green pigments, namely chlorophyll-a and b, however excessive heat, light or air can destabilize the product [8].This destabilization can degrade the chlorophyll product. The structure of chlorophyll is porphyrin macrocycle with four pyrrole rings, while
presence of single isocyclic with pyrrole ring built phorbin structure. There are four carbons and a nitrogen atom in pyrrole ring. The position of nitrogen atoms easily attracts Mg\(^{2+}\) ions for binding. In chlorophyll-b, formyl group take over methyl group in ring, which differentiate it from chlorophyll-a. This structure difference between two classes of chlorophyll makes identification process easier by their peaks at respective wavelength and region (665 for Ch.-a, and 652 for Ch.-b) [8].

The downstream process of cell disruption for microalgal cell to release associated products is necessary, which could be achieved via physical or chemical method [9]. Earlier, soxlet extraction procedure assisted with solvent was famous one, but it is time and energy intensive. The technological development in research and industrial sector introduced the new ways of disruption such as ultrasonication and microwave. These methods do not only rupture the high dense cell very effectively but also conform short duration as well.

The use of ultrasonic as extractant method is of increased interest widely due to high efficiency and shorter time required [10]. The extraction process usually carried out with aid of chemicals such as chloroform, acetone, methanol and ethanol. A normal protocol for chlorophyll extraction use acetone as effective solvent, however this is one of toxic solvent. The solvent selected for extraction process must be less harmful. Normally cell disruption of algal biomass release intercellular products, which comprised of lipids and chlorophylls mixture known as crude extract. Lipids are precursor of biodiesel production, which is not an objective of current study. Various studies conducted on lipid extraction from microalgae but limited data is found on quantification of chlorophylls in crude extract using different solvent. Current study focused on optimization of process and quantification of chlorophylls in crude extract, extracted via solvent assisted ultrasonic technique.

2. Material and Methodology

2.1. Material and Chemicals

A marine microalgae Chlorella sp. was cultivated in 20 m\(^3\) open aerated pond at National Institute of Coastal Aquaculture (NICA), Songkhla, Thailand as shown in Figure 1. The culture growth was attained using CO\((\text{NH}_2)_2\) and 16-16-16 fertilizer (16% N, 16% P and 16% K) as growth medium. Due to open cultivation scheme the other sources such as Light and CO\(_2\) were provided naturally for photosynthetic action. Commercial grade n-hexane, methanol, ethanol and acetone were purchased from ACI Labscan Ltd., while aluminium sulphate was obtained from Saim chemical company Ltd. Thailand.

![Figure 1](image1.jpg)

Figure 1. Marine Chlorella sp. cultivated in 20 m\(^3\) open pond system

2.2. Biomass harvesting and post processing

The cultured biomass attained peak growth phase in 7 days with cell density of 0.8 g/l was harvested using aluminium sulphate as flocculant agent. After harvesting biomass slurry was filtered using cheese cloth to remove any present contamination, filled in 20 L gallons and stored at 4 °C. The stored algal biomass slurry was washed thrice with DI water to remove salinity and pH was recorded as 7.5.

Cleaned biomass slurry was subjected to dewatering using vacuum filtration with GENVAC vacuum pump (PVL 3, 0.5 mbar, Italy). The slurry was stirred gently to enhance filtration rate. The wet paste as shown in Figure 2 was collected in zip locked air tight bags and stored at 4 °C for short period of time before starting extraction. 1gallon (20 L) slurry yields about 1 kg wet paste.
Figure 2. Fresh wet algae paste (10-12% dw solid biomass) after vacuum filtration

Extraction process was performed using ultrasonic bath (Crest power sonic, 45 kHz, CP 2600, USA). Experiment was conducted at different temperature (30-40 °C) and time (60-120 min) values. The Stored wet paste biomass was brought to room temperature firstly, then 10 gm (dw% basis) was placed in 250 ml Duran bottle. Organic solvent methanol: hexane (2:1 v/v) was mixed with sample and stirred for 1 minute. To prevent chlorophyll degradation due to light, sample bottles were covered with aluminium and placed in ultrasonic extraction bath at predefined condition. The extract after different time intervals were taken out and filtered using whatman 4 by vacuum application.

Residual biomass was stored for further processing, while filtrate was evaporated using Heiolph Laborta 4000 rotary evaporator for solvent recovery. The extract obtained after solvent recovery was solid at ambient temperature. This solid was resuspended in methanol initially, instead of solvent mixture used for extraction, because developed simultaneous equation only deals with single solvent. Methanol was selected because of its higher proportion as extractant and have polar nature as well, which can easily dissolve green pigments. Optimum condition for chlorophyll quantity (previously using methanol) was determined, after that extract was dissolved in different solvents such as ethanol and acetone to perform analysis.

2.3. Analysis
The compositional analysis of wet paste biomass was performed according to standard procedures of Association of Official Analytical Chemist (AOAC). The AOAC protocols 991.20, 920.39,942.05 were adopted to find out protein, crude fat and ash. Moisture contents were determined on the weight loss basis at 90-95 °C, while ANKOM 200 analyzer was used to measure crude fiber contents at ADCET, PSU, Thailand. The carbohydrate and energy contents were determined by calculation method.

The UV-spectrophotometer (Agilent 8453, USA) was used for chlorophylls analysis. The sampling quartz of 1 cm was used in spectrophotometry. UV system was calibrated prior to analysis and blank reading was taken. Initially different dilutions 1:10, 1:15, 1:20 and 1:25 ml/ml (sample: solvent) were performed to get clear peaks at full spectrum range (100-800 nm). Further measurements were taken at best found dilution factor. Green pigments (chlorophyll a and b) absorb light in red and blue region. The developed simultaneous equations [Equation (1-3), for methanol, ethanol and acetone respectively] were used to quantify chlorophylls from plants [11].

\[
\begin{align*}
\text{Ch} - a &= 16.29A_{665.2} - 8.54A_{632.0} \\
\text{Ch} - b &= 30.68A_{665.2} - 13.58A_{665.2} \\
\text{Ch} - a &= 13.36A_{664} - 5.19A_{649} \\
\text{Ch} - b &= 27.43A_{649} - 8.12A_{664} \\
\text{Ch} - a &= 12.25A_{663.6} - 2.55A_{646.6} \\
\text{Ch} - b &= 20.31A_{664.6} - 4.91A_{663.6}
\end{align*}
\]
The samples absorbance was recorded at desired wavelength as per above equation and quantity (µg/ml) of chlorophylls were calculated. The total quantity of chlorophyll a and b was determined based on summing up the individual chlorophyll values and modeled as yield.

The $3^2$ (2 factors 3 levels) full factorial response surface experimental design with 2 additional runs at centre point was selected to observe the effects on response (total quantity of chlorophylls). The design of the experiment, parametric analysis (ANOVA) by multiple regression and optimization was performed using Statistica version 10.0. Two factors temperature as $X_1$ (30, 35 and 40 ºC) and time as $X_2$ (60, 90 and 120 min) were coded into three level as -1 (low), 0 (center) and +1 (high). The relationship between coded and actual value is presented in Equation (4).

$$X_i = \frac{x_i - x_o}{\Delta x}$$  \hspace{1cm} (4)

Where $X_i$ is coded value of independent variable; $x_i$ is original factor; $x_o$ is base value at center point and $\Delta x$ is step change between low and high level. The experimental data were fitted according to Equation (5), which is the general form of the proposed model.

$$y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i>j}^{k} \beta_{ij} X_i X_j + \epsilon$$ \hspace{1cm} (5)

Where $y$ is response and $\beta_0$, $\beta_i$, $\beta_{ii}$, $\beta_{ij}$ are linear, quadratic and interaction terms of model. The predictive response was studied for dependent variables and correlation equations were developed.

3. Results and discussion

3.1. Wet paste biomass compositional analysis

The compositional analysis of wet paste was determined by standard AOAC procedures. The composition of different microalgae strains is different, even same strain could have different composition due to diversity in culture. Moisture contents were found 90%, which means only 10% (dw) solid contents of algal biomass available. Fang et al., (2016) reported the findings for Chlorella sp. Wet biomass [12]. It is in normal range with respect to vacuum filtration application to concentrate biomass from 5-15%. Protein, crude fat, ash, carbohydrates and energy (kcal) contents were found 0.76, 0.8, 1.36, 1.59 and 9.43 respectively.

3.2. Ultrasonic extraction and optimization

The full factorial response surface methodology was adopted to design and optimize the ultrasonic extraction experiment. Response surface methodology is one of the most attractive tool for optimization of process [13]. The details of experiment such as coding and actual factors scheme, experimental and predicted response are presented in Table 1. The response fitted model regression analysis (ANOVA) is shown in Table 2. The data shows that experimental and predicted response is in good agreement and residual is minimal. Regression analysis was performed at 95% confidence level.

The model F value is good and making it significant, only 0.01% chance is there that error could be due to some unavoidable factor. Both linear, interaction and squared interaction terms are significant having p-value less than 0.05. Lack of test is non-significant with p-value of 0.91, which confirms the best fit of model. The R-squared of 0.99 very close to 1 is presenting good fit of model as well at 95% confidence.

On the other hand, adjusted and predicted R-squared value of 0.98 and 0.97, which are very close to R-squared value of 0.99 are well justifying the model. The standard deviation of 0.18, and 1.26% C.V leading to reliability of model. By backward elimination as setting confidence level at 0.95 a refined equation (6) in terms of coded factors was developed for response variable.

$$Y = 13.80 - 1.57X_1 - 0.95X_2 + 0.43X_1^2 - 1.17X_1X_2 + 1.12X_2^2$$  \hspace{1cm} (6)
The selected solvent methanol shows high response in extracting these value-added products, as it was confirmed in our preliminary analysis as well. Some studies also reported methanol as consistent solvent for excellent capability of chlorophyll extraction compared to other solvents [14,15].

Response surface and contour plot of factors with respect to yield are given in Figure 3. Both factors showing significant effect on response, at 30 °C yield increased linearly with time increment but at 35 °C and 40 °C response yield trend was found in decreasing order as time increased. Kong et al.[16] study states that chlorophyll extraction via ultrasound increased linear with time at specific temperature, after that it started to decrease. This could be due to fact that chlorophylls start to degrade, if excessive heating applied [17]. The optimum condition was observed 30 °C and 120 min at which recovery was 17.19 (µg/ml). Two trials were performed at optimized condition to check accuracy and found ± 0.02.

Table 1. Full factorial design runs with actual and model predicted response

| Run | Actual Factors | Temperature (°C) | Time (min) | A (665.2) | A (652.0) | X1 | X2 | Experimental | Predicted |
|-----|----------------|-----------------|------------|-----------|-----------|----|----|---------------|-----------|
| 1   |                | 30              | 120        | 0.85      | 0.67      | -1 | 1  | 17.20         | 17.15     |
| 2   |                | 35              | 120        | 0.62      | 0.51      | 0  | 1  | 12.90         | 12.8      |
| 3   |                | 35              | 90         | 0.70      | 0.54      | 0  | 0  | 13.80         | 13.85     |
| 4   |                | 35              | 90         | 0.75      | 0.54      | 0  | 0  | 14.0          | 13.80     |
| 5   |                | 30              | 60         | 0.80      | 0.56      | -1 | -1 | 14.50         | 14.45     |
| 6   |                | 40              | 120        | 0.59      | 0.46      | 1  | 1  | 11.70         | 11.67     |
| 7   |                | 35              | 60         | 0.91      | 0.56      | 0  | -1 | 14.80         | 14.75     |
| 8   |                | 40              | 60         | 0.76      | 0.53      | 1  | -1 | 13.70         | 13.67     |
| 9   |                | 40              | 90         | 0.64      | 0.49      | 1  | 0  | 12.60         | 12.67     |
| 10  |                | 35              | 90         | 0.82      | 0.51      | 0  | 0  | 13.50         | 13.8      |
| 11  |                | 30              | 90         | 0.98      | 0.59      | -1 | 0  | 15.70         | 15.8      |

3.3 Dilution factor and dissolving of methanol/hexane extract in different solvents.

The dilution factor for spectroscopy analysis was investigated and 1:20 ml/ml factor is reasonable to produce clear peaks. Dissolving the methanol/hexane solvent extract in different single solvents (as per simultaneous equations) in methanol, ethanol, acetone was solely based on fact to plug data in available equations, and in case of suitability choice of ethanol (non-toxic) as friendly solvent.

It has been found that dissolving extract in different solvent (instead of extractant) is not good choice, however peaks were good but absorbance was more than 2, which sounds not good, because spectrophotometer linear absorbance range is between 0.1-1. Due to this fact chlorophyll calculated amount and absorbance values of sample diluted in ethanol and acetone are not presented here.
Table 2. Regression analysis (ANOVA) of fitted model.

| Source          | SS   | MS  | F    | df | p         | significance |
|-----------------|------|-----|------|----|-----------|--------------|
| Model           | 22.69| 4.54| 144.82 | 5  | <0.0001   | significant  |
| X_1             | 14.73| 14.73| 470  | 1  | <0.0001   | significant  |
| X_2             | 1.80 | 1.80| 57.61 | 1  | 0.0006    | significant  |
| X_1X_2          | 5.52 | 5.52| 176.25 | 1  | <0.0001   | significant  |
| X_1^2           | 0.51 | 0.51| 16.34 | 1  | 0.0099    | significant  |
| X_1^2X_2        | 1.69 | 1.69| 53.86 | 1  | 0.0007    | significant  |
| X_1 X_2^2, X_2^2|      |     |       |    | Not significant |
| Residual        | 0.16 | 0.031| 0.031| 5  | 0.91      | Not significant |
| LOF             | 0.30 | 1e^-2| 57.61 | 3  | 0.0007    | Not significant |
| Pure error      | 0.13 | 0.063| 0.16 | 2  | 0.91      | Not significant |
| Total           | 22.85|     |      | 10 |           |              |

Precession | $R^2$ | $R^2$ Adj. | Std. Dev | Pred. $R^2$ | C.V %
|-----------|-------|------------|----------|-------------|------|
| 41.9      | 0.99  | 0.98       | 0.18     | 0.97        | 1.26|

Figure 3. Surface and contour plots of fitted response for total chlorophyll yield ((µg/ml)).

4. Conclusion
Current study presents that marine *Chlorella* sp. possess considerable amount of chlorophylls, which could be extracted and purified for wide range applications. High temperature was found not suitable for extraction as it might degrade pigments. Dissolving the extract (methanol/hexane) in methanol was found good, but it’s dissolving in ethanol and acetone was not accurate despite of producing clear peaks. It is concluded that same solvent (methanol in current study because of higher proportion with respect to hexane) utilized for extraction is best for dissolving again for analysis. More work is required in purification and developing simultaneous equation for mixed solvent.
Acknowledgment
The corresponding author is grateful to Graduate School Prince of Songkla University, Hat Yai, Thailand for the provision of Thailand’s Education Hub for Southern Region of ASEAN Countries (THE-AC) scholarship, 2016.

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