A Preliminary Study of Extraction and Purification Processes of Astaxanthin from *Haematococcus pluvialis* as a Natural Antioxidant

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Abstract. *Haematococcus pluvialis* is a species of microalgae which contains a prominent natural antioxidant called astaxanthin. However, the thickness of microalgae cell wall hinders the extraction process. This problem (cell wall destruction) can be overcome by implementing one of the efficient methods, microwave assisted extraction. The purpose of this study is to determine the extraction equilibrium constant and simple experiment data on purification process. The extraction was carried out using acetone as the solvent at 50°C under constant stirring speed which was then followed by refinement process. Thin layer chromatography was also performed to select the suitable eluent for purification that drew upon chromatographic column. Based on the experiment, the best eluent from thin layer chromatography was a mixture of acetone and petroleum ether 1:1 (v/v), with the retardation factor (Rf) value of 0.649 under atmospheric pressure. Likewise the highest astaxanthin purity was 6.451mg/L. Meanwhile, the equilibrium constant was investigated by employing the mathematical modeling.

1. Introduction

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are naturally produced by the metabolism of human body, with pro-oxidative characteristic, and can also destroy the molecular system. In normal human body, the formation of ROS and RNS can be controlled by anti-oxidant to achieve the equipoise inside the body [1]. Air pollution, cigarette smokes and advanced glycation end products (AGES) in diabetes will shift the balance of pro-oxidative results to be oxidative stress [2].

In a very high concentration, ROS can potentially damage the cells and tissues which can increase in degenerative diseases, for instance coronary heart and cancer [3]. Similarly, the excess of RNS can also harm tissues which triggers the chronic inflammatory, stroke and septic shock [4]. To reduce ROS and RNS production, additional of antioxidants from inside and outside body are needed [5].
Antioxidants are chemical substances that can inhibit, reduce or prevent oxidative reaction with other compounds [6-8]. These can be synthesized from chemicals or extracted compounds from natural sources, such as microalgae. However, the most widely used synthetic antioxidants are butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and propyl gallate [9]. Meanwhile, the natural antioxidant can usually be found in fruits, vegetables and meats, for example vitamin C (ascorbic acid), vitamin E (tocopherol), vitamin A (carotenoid), and polyphenol substances such as flavonoid, and anthocyanins (types of flavonoid), lycopene (types of carotenoid) and coenzyme Q10 (also called ubiquitin, protein type) [5]. Despite the benefit given by synthetic antioxidants, these chemicals also have drawbacks, render mutagen and poisoning effects. Therefore this phenomenon leads to the high demand of natural antioxidants [7].

One of microalgae species consisting of high natural antioxidant in type Astaxanthin (ATX) is *Haematococcus pluvialis* (*H. pluvialis*) with the concentration of 0.5-5% dry weight [10-13]. Meanwhile, based on lipid peroxidation method, ATX has an antioxidant activity around 100-500 times as high as Vitamin E [14-15], is stronger than Vitamin C [16], and has a quenching activity of 40-600 times as high as α-tocopherol using singlet oxygen quenching method [17]. Nowadays microalgae have attracted many researchers, to be utilized as the raw material of ATX extraction since it merely requires a small arable area, and has a higher growth rate compared to other crops which is solely 7-14 days [18].

By the same token, a lot of tantamount studies have been performed which were pulled off by Dong *et al.*, 2014, Reyes *et al.*, 2014, Wang *et al.*, 2011, and Krichnavaruk *et al.*, 2008. However, the mathematic modeling of ATX extraction has never been probed beforehand, thus this study was conducted to obtain the extraction equilibrium constant and simple experiment data in purification process which can be employed for scale up process. In addition, this research was also aimed to reduce the usage of hazardous chemicals by substituting chemical compounds which are environmentally friendly.

**Materials and Methods**

1.1. Materials

Dry powder microalgae, *H. pluvialis* were purchased from Rongsheng Biotechnology Co., Ltd, China with a content of 3% dry weight ATX. 98% of ATX standards and 2,2-diphenyl-1-picrylhydrazyl were obtained from Sigma Aldrich, Singapore. Meanwhile acetone, ethanol, methanol, petroleum ether, and silica gel were supplied by Merck.

1.2. Methods

1.2.1. Extraction.

1.2.1.1. Extraction process. The process was carried out by using microwave assisted extraction by mixing as much as 2.5 g of *H. pluvialis* with 250 ml acetone as the solvent into the reactor for about 35 minutes at 50°C. The sample was taken every 5 minutes.

The extracted ATX in microalgae is in the form of free ATX (5%), monoester ATX (70%) and diester ATX (25%) as shows on Figure 1 [19].

![Chemical structure of Astaxanthin (ATX)](image)
For analysis, monoester and diester ATX must be hydrolysed to cut off the ester bound to create free ATX. Hydrolysis was carried out by mixing microalgae extract in sample with 0.02N NaOH alcoholic. The mixture was stored in refrigerator at the temperature of 4°C for 24 hours and placed in dark room at room temperature for 3 hours to complete the hydrolysis process. After the hydrolysis process, ATX was measured by Shimadzu UV/Vis Spectroscopy at the wave length for ATX was $\lambda=478$ nm dissolved in solvent which was same as extraction solvent.

1.2.1.2. Extraction mathematic modeling. Microwave assisted extraction (MAE) is an extraction process where the mass transfer begins by percolating solvents against microalgae simultaneously by utilizing the microwave heat generated from the inside to the outside of the cells. The MAE can increase the extraction ATX from microalgae through the internal heat generation. The mass transfer process between ATX and solvents immediately reached the equilibrium state as shown on Figure 4.

ATX mass balance in microalgae can be written as:

$$-M_1 \frac{dC_{AP}}{dt} = A_{S1} k_1 (C_{As} - C_{A1})$$

where $M_1$ = mass of microalgae (gram), $C_{AP}$ = ATX concentration in microalgae (gram ATX/gram microalgae), $t$ = extraction time (seconds), $A_{S1}$ = contact area between microalgae and solvents ($m^2$),
\( k_{c1} \) = ATX mass transfer coefficient from microalgae to solvents (m/second), \( C_{AI} \) = ATX concentration in solvents (gram ATX/m\(^3\)), \( C_{AS} \) = ATX concentration in solvents at the interface (gram ATX/m\(^3\)).

The correlation between \( C_{AS} \) and \( C_{AP} \) can be expressed by equilibrium constant (K) as follow.

\[
C_{AS} = K \cdot C_{AP} \quad \text{..................................(2)}
\]

1.2.2. Selection of Suitable Solvent. Thin Layer Chromatography (TLC) was used as the method to determine eluent suitable for the purification process using methanol, acetone and petroleum ether as eluents with different compositions.

1.2.3. Purification. Atmospheric Chromatography column was employed as the purification equipment which consists of stationer phase, Silica gel \(_{60}\)PF\(_{254}\) containing gypsum, and mobile phase, TLC result.

1.2.4. Antioxidant Analysis. Antioxidant activity was analyzed using 2,2-diphenyl-1-picrylhydrazyl (DPPH analysis). One mL DPPH was mixed at various concentrations with methanol until the total volume was 5 mL. Afterwards the mixture was shaken and left in the dark place for about 30 minutes and then analyzed using Shimadzu UV/Vis Spectroscopy with the wave length of \( \lambda = 517 \). The scavenging activity was measured as shown in Equation (3):

\[
\text{Radical Scavenging Activity (\%) } = \left( \frac{A_c - A_s}{A_c} \right) \times 100 \% \quad \text{.................(3)}
\]

where \( A_c \) and \( A_s \) denote control and sample absorbances respectively. Control solution was the mixture of 1 mL DPPH and 4 mL methanol. IC\(_{50}\) can be reached in the radical scavenging activity at the value of 50%.

2. Results and Discussion

2.1. Extraction

Quantitative analysis of microalgae extract was difficult to be performed because the extract contains a lot of esters bound to ATX. Therefore, the hydrolysis process should be undertaken in order to cut the ester bounds off, so it could change the RCOOR with –OH groups to create the free ATX by following the hydrolysis process carried out by Sun H., et al [21].

Other than that, the effectiveness of hydrolysis process can be seen from FTIR analysis as shown on Figure 3. FTIR analysis was performed to see the alternating C-O bound to be C-H group. The Figure 3(a) presents that the –log CO is greater than –log CH which means that the hydrolysis process is an effective method to eliminate esters from ATX. From Figure 3(a), it was found that the free ATX concentration dramatically rose along with the increasing of NaOH alcoholic volume with the extract of \( H. \ pluvialis \) volume was twice (\( V=2V_s \)) (Figure 3(a)). The increasing NaOH alcoholic volume to the extract more than third (\( V=3V_s \) and more) tended to decrease the recovery of free ATX. It was probably because ATX degraded to other compound. \( V \) was NaOH alcoholic volume and \( V_s \) was microalgae extract volume.
Figure 3. The effectiveness parameters of hydrolysis process

The effectiveness of hydrolysis process can also be seen from spectrophotometry analysis as shown on Figure 3(b). In the process without hydrolysis process, the free ATX at the first 25 minutes increased from 26 to 29 mg/L as shown on Figure 3(b) with the free ATX recovery was about 8-10%. In the first hydrolysis process, NaOH alcoholic was added into the extract of H. pluvialis at the same volume ratio (V/Vs=1). From the result, free ATX concentration raised to 20% in 5 minutes and increased to 30% in 30 minutes. Meanwhile in the second hydrolysis process, with the ratio of NaOH alcoholic was twice as high as the sample (V/Vs=2), it can be obtained that free ATX increased from 22% to 66% or equal to 44.91 mg/L in 35 minutes. Therefore, from Figure 3(a) and 3(b), it can be concluded that the optimum ratio of NaOH alcoholic to sample in hydrolysis process was 2:1.

The SEM analysis depicts the cell structure before and after extraction process as shown in Figure 4. It can be seen that the cell after extraction was still undamaged because the extraction process was not optimum yet. Hence it needed another process to destroy the microalgae cell walls. Pretreatment with HCL solution probably can damage the microalgae cell wall.

Figure 4. SEM analysis of cells of microalgae

Figure 5 shows the plotting experimental results and simulation data using Matlab and it shows the resemblance, with the Sum of Square Errors (SSE) of 26.9621. So it can be assumed that data from mathematical modeling could represent the data from experiment. By simulating the aforementioned mathematical modeling, the equilibrium constant (K) and mass transfer coefficient (k_{ci}) values were found, 0.5906 gram microalgae/m³ and 0.0329 m/second respectively.
Figure 5. Experimental and simulation results

The values of $K$ and $k_{cl}$ are the important parameters in solid-liquid extraction due to $K$ is used to determine the minimum ratio between solvent and extracted solute, and to design the extraction equipment.

2.2. ATX Purification

As microalgae extract still contained many substances beside ATX, the advanced refining process is needed to obtain pure ATX. Purification process was carried out by using chromatography column. To achieve the optimum process, the most suitable eluents was analyzed by using thin layer chromatography (TLC) process as shown in Figure 6 (a-d).

![Figure 6. TLC results of eluents in purification process](image)

In the above results, each sheet of TLC which has been dropped by the ATX standard and microalgae extract was dipped in the eluent in various concentrations. Subsequently, through the time each sample was eluted upwards by the eluent until it finally stopped at certain points. Since ATX standard was a pure compound, so it did not disperse on the sheet while the process was drawn upon. Whereas microalgae extract consisted of many compound, hence it degraded and diffused into many spots. Those dots which have similar elevation indicate the same chemical substances. Furthermore, the
effectiveness of TLC analysis can be seen from retardation factor (Rf) which represents at range 0.2-0.8. From Figure 6(c,d), it is clearly seen that the spots have the same elevation as ATX standard than Figure 6(a, b). Likewise Figure 6(c) has a Rf value of 0.872, whilst Figure 6(d) was merely 0.649. Eventually it can be concluded that Figure 6(d) shows the most suitable eluent compared to others.

Chromatography column used in ATX purification from microalgae extract consisted of silica gel 60PF254 contain gypsum as the stationary phase whereas the acetone:petroleum ether 1/1 (v/v) was used as the mobile phase.

Other than that, Figure 7 represents the purification products. From number 1 to number 13 (Figure 7(a)), it shows that the liquid became lighter since the concentration of astaxanthin decreased scantily.

From the experiment (Figure 7(b), it can be concluded that the purification was not effective yet because ATX was still contained in each sample. The highest ATX concentration obtained was 6,451 mg/L in the 30-minute purification process.

![Figure 7. Purification result of astaxanthin](image)

(a) Samples were taken every 10 minutes in purification process

(b) Free ATX concentration in each purification process sample

2.2. Antioxidant Activity

The antioxidant activity of microalgae extract was analyzed using DPPH. It is shown that the increase of ATX concentration caused the decrease of IC\textsubscript{50}. In the first 5 minutes extraction time, IC\textsubscript{50} value was 59.85 mg/L (ATX concentration was 32.02 mg/L), while in the next 10 minutes, IC\textsubscript{50} value was only 53.60 mg/L (ATX concentration was 44.12 mg/L). So it can be concluded that the upsurge in 37.79\% ATX was possibly decline IC\textsubscript{50} as much as 11.64\%.

3. Conclusions

Pre-extraction process was needed to maximize the ATX result from microwave assisted extraction. After extraction process, sample should be hydrolyzed with NaOH alcoholic. The optimum free ATX concentration could be achieved in NaOH alcoholic to sample ratio of 2:1. By simulating the aforementioned mathematical modeling using Matlab, K and k\textsubscript{c1} values in the extraction process were found, 0.5906 gram microalgae/m\textsuperscript{3} and 0.0329 m/second respectively.

Antioxidant activity in microalgae extract was analyzed using DPPH. In the increasing of ATX concentration, IC\textsubscript{50} value decreased. It was indicated that ATX had good antioxidant activity. The lowest IC\textsubscript{50} value was 53.60 mg/L. Purification was an important process to obtain pure free ATX from microalgae extract. Chromatography column was used as method in purification process. The eluent was the mixture of acetone and petroleum ether with ratio of 1:1 (v/v) which was resulted from TLC process. The highest ATX concentration obtained was 6,451 mg/L in the 30-minute purification process.
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