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Research Article

Extraction of astaxanthin from *Haematococcus pluvialis* microalgae using a biphasic solvent system

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Abstract

This study focused on the effect of a two-solvent immiscible system consisting of 0.2M H_2SO_4 solution and ethyl acetate - hexane mixture for rapid extraction and recovery of astaxanthin and its derivatives from *Haematococcus pluvialis (H. pluvialis)*. The effect of changing the composition of solvent in the two-phase liquid system on the ability to extract astaxanthin was evaluated and then, the ratio of the organic solvent mixture was optimized. The results under the survey conditions showed that the maximum extraction efficiency reached 95.02 ± 1.88% with the organic solvent volume/microalgae mass ratio was 12 mL/g when hexane/ethyl acetate ratio was 1/1, showcase significantly higher than the single solvent extraction efficiency. The results of the optimal function calculation using the centered combination method gave an expected organic solvent volume/microalgae mass ratio was 9.563 mL/g with the hexane ratio in the organic solvent was 61.31%, the expected efficiency achieved was 83.26%. In addition, the study also showed that the concentration of H_2SO_4 only affects the process of cell wall disruption and have none affecting the dissolution process. Plus, the dissolution reaction of astaxanthin from *H. pluvialis* microalgae is a first-order chemical reaction.

Keywords: *Haematococcus pluvialis, astaxanthin, immiscible phase, central composite design, optimization.*

1. INTRODUCTION

Astaxanthin is a member of the xanthophyll (xanthophyll group of carotenoids) group which is characterized by high antioxidant activity, which is up to 10-fold stronger than the antioxidant of beta-carotene and up to 500-fold stronger than vitamin E [1, 2]. Because of its excellent bioactivity, astaxanthin has been broadly utilized in cosmetics, nutraceuticals and pharmaceuticals due to its strong antioxidant, anti-radical and anti-inflammatory properties.

Astaxanthins originated from natural origins in some crustaceans [3], as well as from several other foods in the world [3] and are found in certain species of other marine organisms such as fish, salmon [4], *Xanthophyllomyces dendrorhous* yeast [5], *Tetradesmus obliquus* microalga [6], and particularly *Haematococcus pluvialis (H. pluvialis)* [7, 8]. Such a high level of accumulation will make *H. pluvialis* desirable animal for research and development in the world scale microalgae industry. Nevertheless, commercial applications of astaxanthin extraction from *H. pluvialis* are still problematic. Cultivating the

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microalgae in the red phase, at the point when astaxanthin has become concentrated, has to be worked under favorable environmental conditions, which results in much higher cultivation costs.

On the other hand, present recovery and extraction methods are not adequately optimized in terms of energy usage or efficiency [1]. As such, new technologies for astaxanthin extraction should contribute to increases in extraction productivity, reduction in production costs and improvement in the economic ability of natural products as a substitute for synthetic chemical products.

A major challenge is the dense and durable cell wall of *H. pluvialis* at a process during the astaxanthin accumulation stage (red phase) that has a lipophilic algaenan-enriched membrane that is bio-statically insensitive and has high resistance to degradation [9]. This property hinders the effective penetration and dissolution of intracellular astaxanthin by traditional extraction solvents (ethanol, isopropanol or saline solution [NaCl]), resulting in prolonged processing periods and ineffective extraction [10, 11]. In addition, microalgae, especially *H. pluvialis* in the red phase, possesses a thick, rigid, resistant to deterioration protective cell wall. However, even physical assistance techniques including ultrasound [13], pressure compression [14], and bead milling [15], which have been used to improve cell disruption efficiency, have limited impact on this in the short time and distance gap in extraction yield or separation of product phase in microalgal [12]. To increase the effectiveness of cell-derivative disruption, other physical techniques including ultrasound [13], pressure compression [14], and mechanical milling [15] are also used to promote cell-breaking capabilities. However, these methods are still limited to one of the two current problems.

More recently, the heterogeneous liquid-liquid biphasic solvent extraction method has been studied as a promising technique that combines the advantage of classical solid-liquid extraction and selective liquid-liquid extraction, as well as the advantage of liquid-liquid extraction [16-19]. In this method, a combination of two immiscible solvents, commonly acidic aqueous solution and hexane, serves as the extraction medium instead of one organic solvent. Typically, a light solvent phase is chosen as the organic phase that can dissolve the target compound, and the acidic aqueous solution serves as the heavy phase that not only hydrolyzes the cell membrane but also hydrates with microalgal debris, causing the mass of the cell to rise. The concentration of the hydrophobic target compounds and the microalgal residue is separated at the same time for the different solvents, after forced agitation is stopped. Astaxanthin has been isolated using biphasic solvent extraction due to its lipid-like properties [20-22]. These studies demonstrate a considerable reduction in energy use and are as good as traditional methods of extraction. Because the solvents used for the whole process have a toxic side, the methods have limited usage in food production. However, the toxicity issue in this is reduced under controlled conditions in acidic and organic solvent preparation.

Astaxanthin is very soluble in solvents with low polarity (acetone, ethyl acetate, and dichloromethane), poorly soluble in polar solvents (water and alcohol), and insoluble in solvents without polarity (hexane) [2]. Conversely, acetone is miscible with water among non-polar solvents and cannot condense into a biphasic liquid system and dichloromethane is highly toxic, thus ethyl acetate is the one substance most appropriate. Nevertheless, ethyl acetate's solubility in water is as high as 10% [23], thereby, a non-polar component for the solution can allow a more complete phase separation. The process of the production of astaxanthin from the microalgae *H. pluvialis* and extraction of the microalga from it using H_2SO_4 solution and ethyl acetate in hexane combined with hexane has been investigated in this investigation. The volume ratio on organic solvent mixture (mL) on microalgal biomass (g) from 8 to 12 were investigated for the hexane present in organic solvent was found to be 0 - 100% [22]. The solution extraction efficiency of astaxanthin is optimized in comparison to solvent-to-biomass ratios and percentage of hexane in the organic solvent mixture (by volume) via the response surface methodology on the Design-Expert software. After the best extraction parameters are found, the best extraction time with different concentrations of H_2SO_4 is calculated and optimized using a Gaussian function through OriginPro 2018 software.

Its objective is to provide a process to extract astaxanthin at low-energy and effectively to *H. pluvialis* microalga through a heterogeneous liquid-liquid biphasic solvent system. The method is optimized by central composite design (CCD) and implemented in Gaussian models to define its suitable parameters like organic solvent/biomass ratio (mL/g), hexane ratio of the solvent mix, H_2SO_4 concentration, and reaction time. The anticipated results are expected to facilitate the transfer from in-class experimental work to industrial

application and open the direction for broad-spectrum potential application for biphasic extraction technologies in addition to carotenoids deriving from diverse biotic sources.

2. MATERIALS AND METHODS

2.1. Chemicals

Sulfuric acid (H_2SO_4) was given with a purity of 95 - 98%, hexane with a purity of ≥ 98.5%, ethyl acetate with a purity of ≥ 99.7%, HPLC-grade astaxanthin (a purity of 97.2% - batch: 0000397634) was supplied by Sigma-Aldrich. Deionized water with conductivity < 5 µS/cm was provided by Hanna Instruments. The raw material used was commercial *H. pluvialis* microalgae produced by ABR Joint Stock Company (2.11% astaxanthin, ≤ 5% moisture).

2.2. Experimentation

2.2.1. Calibration curve preparation

A 50 mg aliquot of the astaxanthin standard was dissolved in 250 mL of ethyl acetate in a 500 mL volumetric flask. Ethyl acetate was then added to the mark and a stock standard solution was prepared with a concentration of 100 ppm. The working solution was prepared by diluting the stock solution with ethyl acetate to 50 ppm, 25 ppm, 10 ppm, 8 ppm, 5 ppm, 2 ppm, and 1 ppm. The absorption from 350 to 700 nm and the value of the maximum absorption wavelength (λmax) were measured while scanning standard solutions. After that, the λmax was established and used to determine the absorbance of the standard solution, and based on linear regression between concentration and absorption value, a calibration curve was set up. This calibration curve was applied to determine the percentage of astaxanthin from the extraction samples to evaluate the appropriateness and accuracy of the analytical approach on the extracted sample.

2.2.2. Determine the extraction efficiency based on the solvent-to-biomass volume ratio

An accuracy of 1.000 ± 0.010 g of *H. pluvialis* microalgal biomass was weighed in a 50 mL conical flask. Followed by 10.0 mL of a 0.2% H_2SO_4 solution, the mixture was stirred at 600 rpm for 80 min to disrupt the cell architecture. Then a known volume of ethyl acetate and hexane solvent mixture (based on the aqueous phase volume) was added. The extraction of astaxanthins was done by stirring for 120 min. The resulting mixture was added to a separating funnel and allowed to settle for 30 min for phase separation. The aqueous phase, including algal detritus, was collected. The dry algal residue was obtained by vacuum evaporation (60°C, 50 kPa) for 48 h. This residue was then analysed for the presence of any residual astaxanthin.

2.2.3. H_2SO_4 concentration and extraction time were investigated

A series of H_2SO_4 solutions with concentrations from 0.1 - 1.0% were prepared as extract solutions. These H_2SO_4 solutions were added to a 50 mL conical flask containing 1.000 ± 0.010 g of *H. pluvialis* microalgae, followed by a solvent mixture of 6.2 mL hexane and 3.3 mL ethyl acetate. The system was thoroughly stirred. The mixture was then centrifuged at each time point for phase separation. An upper organic layer containing extracted astaxanthin was obtained. The remaining aqueous phase with microalgal residue was sonicated for 20 min and then vacuum-dried at 60°C and 50 kPa for 48 h to obtain a dry residue for subsequent residual astaxanthin analysis. Extractions where the organic layer remained clear, indicating no astaxanthin dissolution, were deemed unsuccessful, and the extraction efficiency was recorded as 0%.

2.2.4. Determination of Astaxanthin content and extraction efficiency

Astaxanthin content in *H. pluvialis* was determined based on an adapted method of A. Lababpour and C. Lee [24]. In short, 0.500 g of dried microalgal residue obtained during extraction studies was combined with 100 mL ethyl acetate and sonicated for 10 min. After centrifugation and solids removal, 1 mL of the supernatant was diluted to 25 mL with ethyl acetate, homogenized by sonication (10 min), and analyzed by UV-Vis spectrophotometry (SP-UV1100, DLAB) at 480 nm. Samples with absorbance outside the calibration range were diluted or concentrated.

The astaxanthin content (E_x, % w/w) in the unextracted *H. pluvialis* microalgal residue is calculated using the following formula (1):

$$E_x = \frac{C}{2} \times k \quad (1)$$

in which: E_x is astaxanthin concentration in the microalgal biomass, expressed in grams of astaxanthin per gram of dry biomass (% w/w). C is the concentration of astaxanthin in the ethyl acetate solution, measured in milligrams per liter (mg/L) and determined via spectrophotometric absorbance. k is the enrichment or dilution coefficient.

The extraction efficiency is determined according to formula (2):

$$H = \frac{E_0 - E_x}{E_0} \times 100\% \quad (2)$$

in which, E_0 is the initial astaxanthin content in the original microalgae sample, E_x is the astaxanthin content in the microalgae after the extraction process.

2.2.5. Data processing and optimization of extraction conditions based on solvent-to-biomass ratio

The results (Section 2.2.2) will be presented as the mean ± standard deviation. Statistical significance will be assessed using one-way analysis of variance (ANOVA). Optimization and regression model will be performed via a Central Composite Design (CCD) using Design-Expert 11 software. The statistical validity of the model will be determined based on p-values, with a p-value of less than 0.05 considered significant. Concurrently, the coefficient of determination (R^2) will be used to evaluate the goodness-of-fit of statistically significant models, aiding in the selection of the most appropriate descriptive function. The objective is to identify the optimal hexane ratio and operating parameters to maximize extraction efficiency.

3. RESULTS AND DISCUSSION

3.1. Results of calibration curve and evaluation of analytical capabilities with samples

Figure 2 shows UV-Vis absorbance spectra for standard astaxanthin solutions of ethyl acetate over the 350 -700 nm wavelength range. At levels lower than 5 ppm, the spectrum shows an absorption peak at 480 nm. By comparison, at 8 and 50 ppm, a bathochromic shift of the main peak was detected to about 463 nm and the emergence of shoulders was shown. This change in spectral change is typical of the influence from the molecular aggregation or self-absorption effects on molecules at high concentrations.

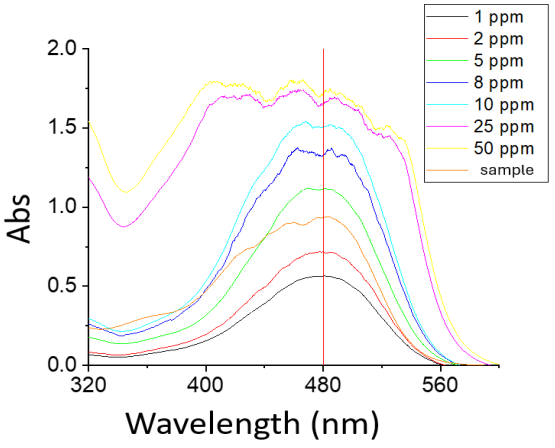

***Figure 1**. UV-Vis absorption spectra of astaxanthin in ethyl acetate at varying concentrations*

Comparison of the spectra of **Figure 1** shows a uniform concentration-dependent spectral shift between different concentrations, with a clear concentration dependent shift. At smaller concentrations (1-5 ppm), the spectrum shows a single peak absorption maximum, which is clearly defined (480 nm). At (8-50 ppm) concentration, this peak undergoes hypsochromic (blue) shift to 463 nm, together with the formation of various secondary peaks or shoulders (i.e. molecular aggregation or self-absorption effects).

The equation for the standard calibration curve was establied by measure the absorbance of standard solutions (**Figure 2** and **Table 1**), the equation is:

$$Abs = 0.0950x + 0.5572 \quad (3)$$

Where *Abs* is the measured absorbance and *C* is the astaxanthin concentration in the solvent (mg/L).

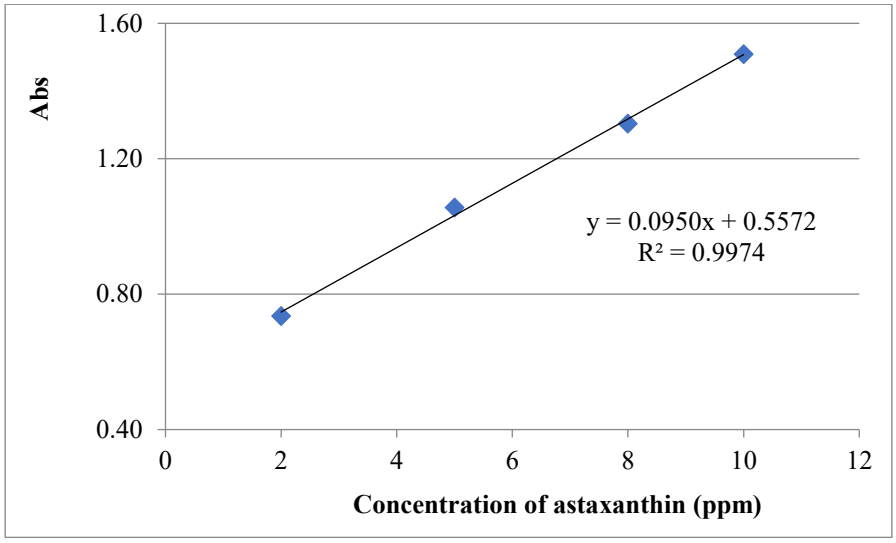

Figure 2. *Astaxanthin calibration curve*

The mean absolute deviation of the calibration data, as illustrated in **Figure 3** and described in **Table 1**, was 3.45%, within the acceptance criterion for analysis (< 5%). Among these regression models, the curve fitting for 2-10 ppm concentration range had a lower deviation, with all points showing less than 6% error. The first calibration curve, in contrast, displayed deviations of over 10% even at 2 ppm. Thus, for all tests carried out in the present research the calibration curve built from the 2 to 10 ppm concentration range was taken.

Table 1. *Absolute deviations of measured values from the calibration curve at specified concentrations*

Concentration (ppm)	Abs	C' (ppm)	RSD%
10	1.509	10.0189	-0.19%
8	1.304	7.8611	1.74%
5	1.056	5.2505	-5.01%
2	0.736	1.8821	5.89%

The absorption spectrum of *H. pluvialis* extract (at 430 nm and 459 nm) showed additional peaks, but the dominant absorbance peak was 480 nm. This in turn, led to the application of Equation (3) in determining the astaxanthin contents. The resulting value (2.06% w/w) closely matched the manufacturer-reported specification (2.11% w/w, based on an HPLC-UV), therefore confirming the accuracy of the spectrophotometric method for a reliable spectrophotometric analysis.

3.2. Results of optimization of extraction efficiency based on biomass-to-solvent ratio and hexane-to-ethyl acetate ratio

The extraction efficiency of different solvent ratios is presented in **Table 2**. Ethyl acetate is excellent for astaxanthin solubility, but its effectiveness is greatly reduced based upon its partial miscibility with water (~8% w/w) [23]. Consequently, much of the ethyl acetate fraction will be lost to water, especially at low solvent volumes. Therefore, in all scales evaluated, pure ethyl acetate presented similar extraction efficiencies to that of hexane although their solvation property was different. The efficiency improved significantly and the results favored a mixed solvent system. The higher proportion hexane in the mixture improved its phase separation which in turn decreased the removal of ethyl acetate from the aqueous phase. The combination of the two solvents also contributed to an increased yields due to their inherent selectivity. At optimized conditions, this combination can achieve extraction efficiencies of more than 90% which may be considered as an improvement compared to earlier single-solvent techniques.

Table 2. *Extraction efficiency as a function of solvent-to-biomass ratio and hexane content in the organic phase.*

No.	Solvent-to-biomass ratio (mL/g)	% of hexane in organic mixture	Extraction efficiency (%)
1	8	100	36.97 ± 6.40
2	9	100	40.37 ± 5.91
3	10	100	44.95± 6.38
4	11	100	55.37 ± 3.90
5	12	100	58.23 ± 5.92
6	12	66.67	93.11 ± 1.87
7	11	54.55	93.03 ± 1.74
8	12	50.0	95.11 ± 0.33
9	10	60.0	89.59 ± 2.69
10	11	45.45	95.02 ± 1.88
11	10	50.0	92.75 ± 1.16
12	9	50.0	88.59 ± 2.17
13	8	50.0	79.15 ± 3.40
14	10	40.0	90.61 ± 1.43
15	12	33.33	89.63 ± 1.70
16	8	0	51.06 ± 3.65
17	9	0	56.35 ± 4.80
18	10	0	64.02 ± 5.59
19	11	0	69.37 ± 1.44
20	12	0	68.07 ± 0.47

This result is notable compared to the available approaches. It beats the 74.0 ± 4.0% yield obtained from acetone with 1:100 solid-liquid ratio with microwave manipulation as mentioned above [26]. It is also approximately twice as effective as a sequential approach with 1N HCl, followed by acetone (1:20 formula), which yielded 73.2 ± 1.0% [27]. It should be noted however that these comparative studies calculated efficiency based on relative HPLC signal intensity with no reporting of the initial astaxanthin content in raw microalgae.

3.3. Optimizing the hexane ratio for the extraction process

Table 3 shows the ANOVA analysis results of response surface model, including polynomial and interaction terms for the investigated factors.

Table 3. *ANOVA for the quadratic polynomial model to optimize extraction parameters*

Model	P-value	R^2
Linear regression	0.0973	-0.0521
2FI	0.7732	-0.2838
Quadratic	**< 0.0001**	**0.9203**
Cubic	0.0106	0.9066
Quartic	0.2312	0.6975
Fifth	0.0344	0.9387

Analysis of **Table 3** indicates that the quadratic model is statistically significant ($p < 0.05$) and demonstrates a strong fit, as evidenced by a coefficient of determination (R^2) approaching 1.0. Consequently, the quadratic model was selected to generate the response surface depicted in **Figure 3**, as defined by Equation (4).

$$H = -0.308 + 5.792 \times \mathrm{A} + 1.318 \times \mathrm{B} - 0.014 \times \mathrm{B}^2 \quad (4)$$

In which: H - extraction efficiency (%); A - the solvent-to-biomass ratio (mL/g); B - % hexane in organic mixture (%, V/V)

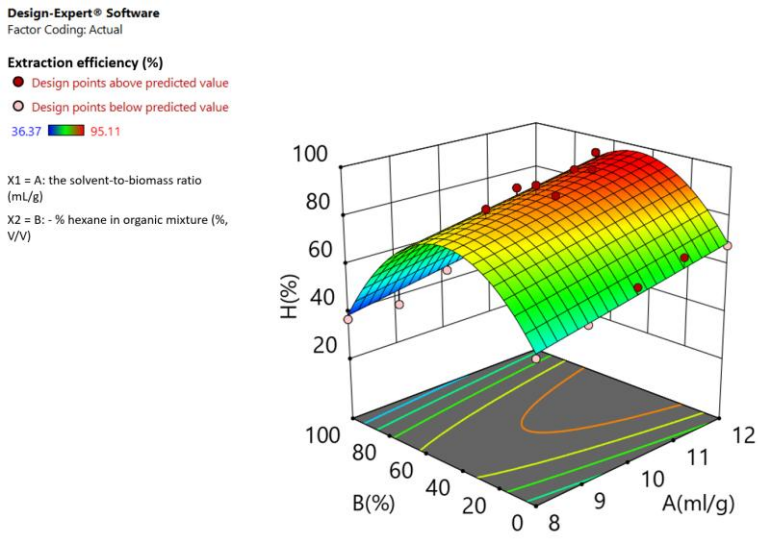

Figure 3. *The surface response describes the relationship among solvent-to-biomass ratio (A), % hexane in organic mixture (B) and extraction efficiency*

Based on the mean absolute deviation relative to the data in **Table 4**, this model fit demonstrates reasonable accuracy, with an average absolute deviation of 4.99%. Therefore, the derived equation can be accepted as a valid basis for optimizing the extraction parameters.

Table 4. *The absolute deviation at various conditions of solvent-to-biomass ratio and % hexane in organic solvent*

No.	Solvent-to-biomass (mL/g)	% hexane in organic mixture	Predict extraction efficiency (%)	Absolute deviation	RSD (%)
1	8	100	37.83	-0.86	-2.31%
2	9	100	43.62	-3.25	-8.04%
3	10	100	49.41	-4.46	-9.92%
4	11	100	55.20	0.17	0.31%
5	12	100	60.99	-2.76	-4.75%
6	12	66.67	94.84	-1.73	-1.86%
7	11	54.55	93.64	-0.61	-0.66%
8	12	50.0	100.10	-4.99	-5.24%
9	10	60.0	86.29	3.30	3.68%
10	11	45.45	94.39	0.63	0.67%
11	10	50.0	88.51	4.24	4.57%
12	9	50.0	82.72	5.87	6.63%
13	8	50.0	76.93	2.22	2.81%
14	10	40.0	87.93	2.68	2.96%
15	12	33.33	97.57	-7.94	-8.86%
16	8	0	46.03	5.03	9.86%
17	9	0	51.82	4.53	8.04%
18	10	0	57.61	5.41	8.58%
19	11	0	63.40	5.97	8.60%
20	12	0	69.20	-1.13	-1.65%

The model obtained shows that the extraction efficiency clearly shows a linear relationship with the solvent-to-biomass ratio. This is explained by the fact that most conditions studied did not meet the optimal predicted condition, so almost the solvent volume/solid mass ratio was linear. On the other hand, hexane content and efficiency are distinctly quadratic. Indeed, a clear parabolic trend is indicated by the experimental range of the factor, meaning that hexane proportion is optimal as shown from the entire span. This agrees with the high superiority of the solvent mixture in comparison to either pure solvent. The optimal extraction parameters are estimated using the model fitting as a solvent-to-biomass ratio of 9.56 mL/g and a hexane content of 61.31% in the organic phase, with a maximum desired efficiency of 83.26%.

3.4. Results determining extraction efficiency depend on extraction time using optimized solvent parameter

The extraction efficiency over the time of extraction was presented in **Figure 4**. Among 4 investigated concentrations of acid, the extraction efficiency of 0.1% H_2SO_4 was 0 that mean there is no astaxanthin was extracted from *H. Pluvialis*. At the concentration of 0.2% and 0.5% of H_2SO_4, the extraction efficiency significantly increased over the time of extraction. This phenomenon indicates that the extraction efficiency did not reach the saturation situation. Interestingly, at 1.0% of H_2SO_4, the extraction efficiency divided into 3 phases. During the first 50 min, the extraction efficiency maintained nearly 0% because of the degradation effect of the microalgae membrane of acid. The second phase was time for astaxanthin initial distribute and solute into extract solvent. Therefore, the extraction efficiency jumped up to nearly 50% after 1h extraction and rapidly increased into 80% at 100 min. After second extraction time, the extraction efficiency reached to saturation situation and maintained above 80%.

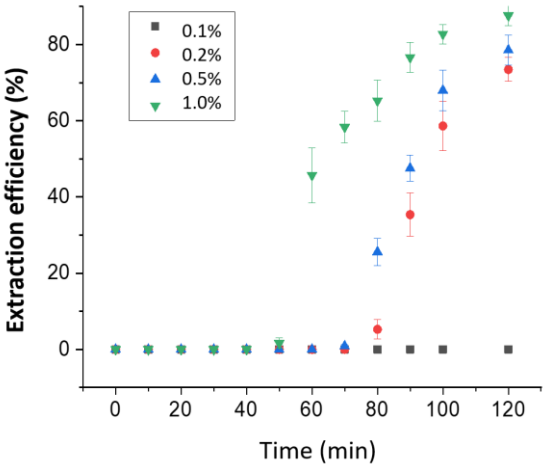

***Figure 4**. Extraction efficiency astaxanthin from H. Pluvialis over extraction time*

The extraction kinetics were obtained as presented in the first-order reaction model (**Figure 5)**. Fitted rate constants for 0.2%, 0.5% and 1.0% H_2SO_4 concentrations (-0.029 min^{-1}, -0.032 min^{-1}, and -0.032 min^{-1}) were respectively. The matching frequency of these rate constants between acidic concentrations suggested H_2SO_4 has a partial effect on disruption time of the first-stage cell wall but does not determine the rate of astaxanthin dissolution after that.

The extraction kinetics were successfully described by a first-order reaction model, as shown in **Figure 5**. The fitted rate constants for the 0.2%, 0.5%, and 1.0% H_2SO_4 concentrations were -0.029 min^{-1}, -0.032 min^{-1}, and -0.032 min^{-1}, respectively. The equivalence of these rate constants across the tested acid concentrations indicates that while H_2SO_4 concentration influences the initial cell wall disruption time, it does not directly govern the subsequent rate of astaxanthin dissolution.

From the above results, the extraction efficiency depends on time and is given by the equation (5):

$$Ln(100 - E) = -0.032 \times t + Ln100 \quad (5)$$

where t is the reaction time in minutes and is calculated from the start of the dissolution process.

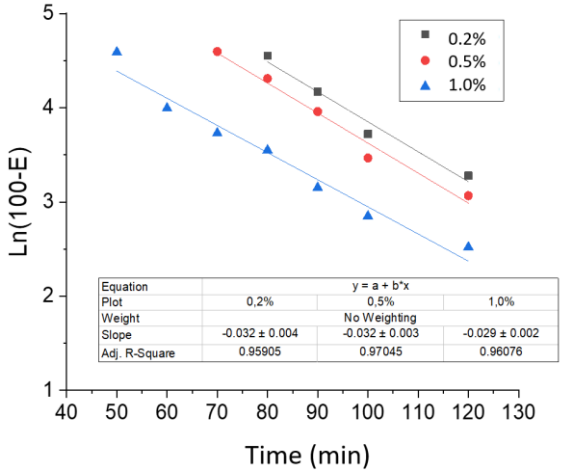

Figure 5. *The extraction efficiency of astaxanthin at various H_2SO_4 concentrations using a first-order reaction rate equation.*

These results, although present, are not without constraints where they cannot quantify the effect of H_2SO_4 concentrations on cell membrane erosion (or destruction) rates. The limitation of such a process stems from the fact that there is currently no instrument capable of determining (directly) and not only relatively evaluating the rate of erosion of the membrane.

4. CONCLUSIONS

This study indicates the technical and economic viability of extracting astaxanthin from *H. pluvialis* using a biphasic liquid system (H_2SO_4 - ethyl acetate - hexane). The separation of phase as well as of phases allows efficient astaxanthin removal and effectively separates microalgal remainders from solvent, and minimizes energy input (of downstream industrially operated solid-liquid separation).

A maximum extraction yield of 95.02 ± 1.88% was obtained with an organic solvent vs biomass ratio of 12 mL/g and a 50% (v/v) hexane solution. However, under optimal conditions for cost benefit and solvent efficiency, the model calculated a better (9.56 mL/g) ratio with 61.31% hexane and an expected efficiency effect of 83.26%.

Also the extraction kinetics were on first-order reaction model. Although H_2SO_4 concentration made the initial disrupt time for the cell wall, it did not change the first dissolution rate constant of astaxanthin to the organic phase thereafter. While preliminary results are encouraging, it remains to assess solvent toxicity, recyclability, and even wastewater treatment. However, this biphasic strategy has a promising scalability for the efficient extraction of additional lipophilic carotenoids from diverse microalgal sources.

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