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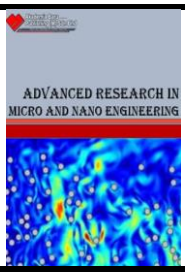

Production of Omega-3 Fatty Acids and Astaxanthin from *Chlorella vulgaris* and *Haematococcus pluvialis* Cultivated in Chicken Manure Medium

Fatin Fazira Abd Karim[1], Shaza Eva Mohamad[1,*], Koji Iwamoto[1]

[1] Faculty of Malaysia-Japan International Institute of Technology, Universiti Teknologi Malaysia, Jalan Sultan Yahya Petra, 54100 Kuala Lumpur, Malaysia

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ABSTRACT

Chicken manure contains a high concentration of nitrogen and phosphate, which can be used to replace microalgae commercial medium. Cultivating microalgae by using chicken manure as the nutrients can reduce culture cost. The aims of this research are to study the effect of different concentrations of chicken manure on the growth of algae, *Chlorella vulgaris* and *Haematococcus pluvialis*. The concentration of omega-3 fatty acids in C. vulgaris and astaxanthin in H. pluvialis was determined by cultivating *C. vulgaris* and *H. pluvialis* in Bold Basal medium and Rudic`s medium respectively and compared with chicken manure medium (CM). The highest concentration of omega-3 fatty acid was obtained in *C. vulgaris* 14.8% in 10% of CM due to depletion of nitrogen which causes the increase of fatty acid production in the microalgae. In *H. pluvialis*, 50% of CM gave the highest concentration of astaxanthin produced (0.082 mg/mL) in *H. pluvialis* due to low concentration of phosphorus in the medium for astaxanthin production. In comparison to nitrate, phosphorus concentration had a more significant impact on astaxanthin synthesis in *H. pluvialis*. In this research, effective culture of *C. vulgaris* and *H. pluvialis* were shown using nutrients i.e., CM instead of commercial medium to obtain the valuable products.

1. Introduction

Microalgae are aquatic plants that grow by consuming the nutrients in their environment and the energy provided by the sun [1]. It has been demonstrated to be a long-term, sustainable source of biomass and oils for fuel, food, feed, and other co-products [2].

Algae can be grown in fresh water or seawater, depending on the species [3]. There were numerous applications for microalgae, including cosmetics, animal feeds, and supplements. Previous study found that *Chlorella* and *Spirulina* species were used as health-food supermarkets and the other species like *Tetraselmis*, *Nannochloropsis* and *Scenedesmus* were used as animal feed [4]. In

* *Corresponding author.*
E-mail address: shaza@utm.my

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this research, the microalgae can be used in aquaculture systems since the medium of cultivation was chicken manure.

The researchers investigated microalgae as nutrients substitutes in fish feeds due to nutritional drawbacks and low fillet consistency. Microalgae have been considered alternatives nutrients in aquaculture as it is rich in amino acids, minerals, vitamins, and long-chain n-3 fatty acids [5].

Two types of microalgae were selected for this study, *Chlorella vulgaris* and *Haematococcus pluvialis*. Omega-3 fatty acids is a source of fatty acids that can be obtained in *C. vulgaris* [6]. According to Yaakob *et al.,* nitrogen and phosphate were the essential mains components in a medium for algae growth [7]. As a result, this research emphasizes the amount of nitrogen and phosphate necessary for microalgae culture and the advantages of nitrogen and phosphate in enhancing the biomass productivity of microalgae. Plant waste and animal waste were two promising alternative mediums.

Chicken manure can be used to substitute the typical culture medium since it is rich in nutrients (e.g., nitrogen and phosphate) [8]. Chicken manure has long been a traditional organic alternative source of fertilizer that is more cost-effective than chemical fertilizers. It also reduces manure pollution caused by improper disposal, allowing much-needed nutrients to be recovered and re-used [9]. Chicken manure was a good choice of source because it is readily available, dissolved rapidly and had a well-defined composition [10].

2. Methodology

2.1 Preparation of Chicken Manure Medium

The chicken manure was obtained from Ukay Nursery, Setiawangsa. The chicken manure medium was prepared by mixing the 200 grams of chicken manure with distilled water (1L) in a beaker. The mixture was gently shaken for 3 times and was kept overnight at 4˚C. The supernatant was collected using filtration process with 20 um of pore size mesh of filter paper was used. Then, the media were prepared at are 10%, 20%, 30%, 40% and 50% of concentration chicken manure stock with tap water. The stock of nutrient was sealed and keep in a bottle at temperature of 4˚C.The medium will not be sterilized because of the autoclaving process can cause the degradation of nutrients inside the chicken manure medium [11].

2.2 Cultivation Conditions

The total volume of cultivation of microalgae was 250 mL with inoculum of 25 mL. The *C. vulgaris* and *H. pluvialis* were cultivated until stationary phase. The temperature at 25 °C was used and maintained by monitoring the temperature using thermometer. The white LED light was used, and the light intensity of the culture was maintained at ±60-70 µmol m^{-2} s^{-1} during cultivation [8]. The light intensity was monitored using light meter. The CO_2 tank was set up and 2% of CO_2 was adjusted using flow meter. The CO_2 was continuously added until the end of cultivation, the installation of CO_2 helps to increase the growth of microalgae [12].

2.3 Analysis Of Total Nitrogen and Phosphorus

The main components of nutrients in growing microalgae were nitrogen and phosphorus. The total nitrogen and phosphorus were determined using standard method which were Persulfate Digestion Method and Molybdovanadate with Acid Persulphate Digestion Method respectively.

2.4 Gas Chromatography Analysis for Omega 3

The gas chromatography and mass spectrometer detectors were quantified and identified the FAMEs in sample. The carrier gas which helium will be used in a spitless mode with continual flow rate (7.10 mL/min) [13]. The diameters of 30 m x 0.25 mm x 0.25 µm of column SH-RxiTM 5Sil MS was used. The temperature was set up to 100°C at initial stage for 1 minute. Then, it continuously increased up to 200°C (1 minute) and 250 (7 minutes) at ramping rate of 25°C/min 4°C/min respectively [15]. The internal standard heptadecanoic acid (C17:0) was used and compared with the peak area of total FAME obtained from GCMS analysis. The FAMEs content was calculated using Eq. (1) [14].

$$\text{FAME content}, \% = \frac{A_{FAME}}{\sum A - A_{C17}} \times 100\% \quad (1)$$

Where FAME is a peak area of fatty acid methyl esters, ∑A is total peak area of FAME chromatogram and A_{C17} is a peak area of the internal standard (C17:0).

2.5 Extraction of Astaxanthin in H. pluvialis

The *H. pluvialis* cells were collected after the cells turn to red and 50 mL of cultures were centrifuged at 25 °C for 5 minutes at 12000 rpm. Then, after the supernatant was discarded, the pellet was stored at -80 °C for 1 day. The freeze drying was introduced to remove the excessive moisture inside the pellet. After that, the pellet was transferred to centrifuge tube and the sodium acetate was added for 1mL. The sample was centrifuged at 5000 rpm and the supernatant was transferred to cuvette for spectrophotometer analysis. The analysis of astaxanthin using spectrophotometer was done at 432 nm of wavelength [16].

3. Results

3.1 Nutrient Content in Commercial and Chicken Manure Medium

The contents of the chicken manure medium were measured as explained in section 3.2 using the Hach`s method. According to Yaakob *et al.,* nitrogen and phosphorus are necessary macronutrients for microalgae growth [7]. Thus, this research focused on these two compositions by observing their effect on the growth of microalgae. 10%, 20%, 30%, 40% and 50% of chicken manure (CM) were prepared as mentioned in section 2. The results of total nitrogen and total phosphate from the different percentages of CM were as shown in Table 1.

Table 1
Microalgae growth mediums and the concentration of total nitrogen (TN) and total phosphate (TP)

Concentration	TN (mg/L)	TP (mg/L)
Bold Basal Medium (BBM)	11.0	9.4
Rudic Medium (RM)	42.5	10.6
10% of CM	1.0	1.9
20% of CM	9.0	4.2
30% of CM	14.0	7.3
40% of CM	26.0	10.1
50% of CM	30.0	13.6

3.2 Analysis of Omega-3 Fatty Acids

There are three types of omega-3 fatty acids, which are ALA (Alpha Linolenic Acid) (C18:3), EPA (Eicosapentaenoic Acid) (C20:3) and DHA (Docosahexaenoic Acid) (C22:3). These omega-3 fatty acids are abundant in microalgae such as *Dunaliella salina* and *Spirulina* [6]. These three types of omega-3 fatty acids were expected to be obtained in the GCMS analysis in this study. However, during the analysis, only ALA was detected as the omega-3 fatty acid source in *C. vulgaris*. The analysis of omega-3 fatty acids was conducted at Shimadzu Malaysia Sdn. Bhd. Figure 1 shows the composition present during analysis of GCMS.

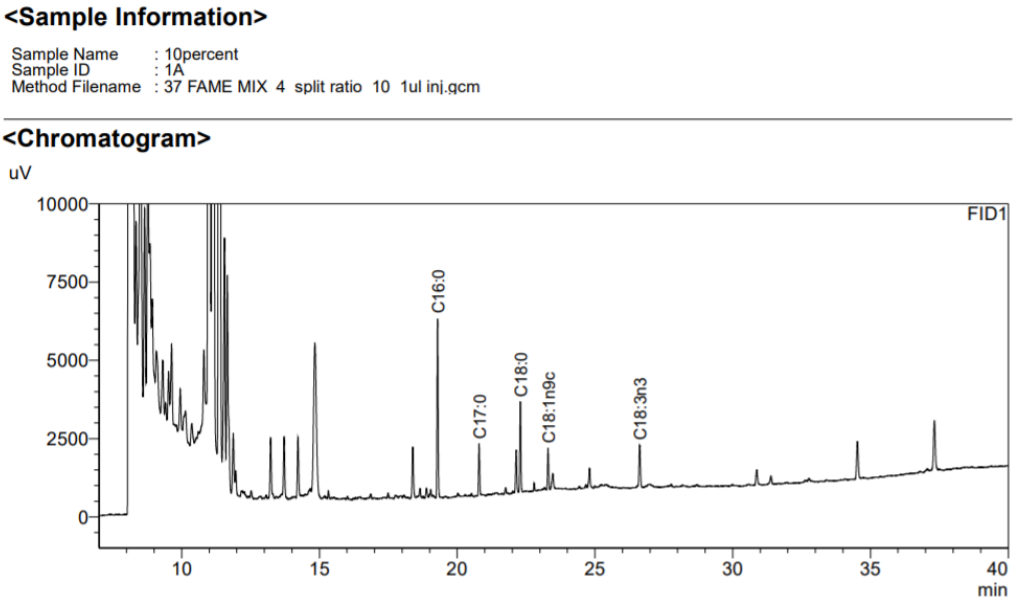

Fig. 1. The fatty acid composition present in 10% of CM analysis

As shown in Figure 1, there are four types of fatty acid composition determined from GCMS analysis. The purpose of this research is to observe and compare the amount of omega-3 fatty acids in commercial and chicken manure mediums. Table 2 shows the types of fatty acids that have been detected in 10% of the CM analysis and their functions.

Table 2
Types of fatty acids found in 10% of CM

No.	Types of fatty acids	Benefits
1.	Omega-hydroxy phytoceramides (ω-oh-phytoceramide/C16)	Enhanced the integrity and accelerate the recovery of damaged skin barrier function [17]
2.	Stearic Acid (C18:0)	Reduced blood pressure, improved heart function, and reduced cancer risk [18]
3.	Oleic Acid (C18:1n9c)	Commonly used for preventing heart disease and reducing cholesterol [19]
4.	Alpha-Linolenic acid (C18:3)	A moderately lower risk of cardiovascular disease [11]

Based on Table 2, fatty acids detected in the medium showed various benefits to human health. Hence, it will be an added value to be introduced in the aquaculture industry. However, further study needs to be encountered on this issue. The quantification of omega-3 fatty acids was calculated based on the quantity of FAME measured by comparing the peak area of the total FAME chromatogram with heptadecanoic acid (C17:0) as an internal standard.

The FAME content was calculated in percentage, whereas the peak area of fatty acid was divided with the total peak area of the FAME chromatogram and the peak of internal standard. The calculation was based on section 2 (Eq. (1)). The peak area of the FAME chromatogram was generated automatically by the GCMS equipment. Table 3 shows the percentage of fatty acid composition that has been calculated.

Table 3
The percentage of fatty acids composition

Types of medium	Fatty acids composition (%)			
	C:16	C18:0	C18:1n9c	C18:3n3
BBM	61.6	34.3	4.2	0.0
10% of CM	48.7	24.9	11.6	14.8
20% of CM	56.1	38.3	1.8	3.8
30% of CM	54.1	33.7	3.2	9.1
40% of CM	55.8	37.7	1.7	4.8
50% of CM	52.4	37.3	4.9	5.5

From Table 3, 10% of CM shows a high percentage composition of omega-3 fatty acids, followed by 30% of CM with 14.8% and 9.1%, respectively. As stated in a study, the amount of depletion amount of TN contributes to the high amount of fatty acid composition. A previous study found that the growth of *C. zofingiensis* was severely inhibited by nitrogen deprivation in one investigation [7].

Furthermore, microalgae with slow growth rates in nitrogen-deficient environments tend to accumulate more saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) in neutral lipids. On the other hand, it leads to a more significant accumulation of polyunsaturated fatty acid (PUFA). Culture media with a high nitrogen and phosphorus content are better for microalgae growth rates and biomass output. However, nutrient-limited conditions will be efficient for lipid and fatty acids generation [7].

Based on another study, biomass production dropped when the nitrate concentration in the medium decreased while the lipid content increased. In the culture with 0.05 g L1 KNO_3, which is one-fourth of the basal nitrogen supply concentration, the highest lipid accumulation of 26% was observed [20]. Thus, 10% of CM seems to be the best concentration for high lipid accumulation since it contains less nitrogen and phosphate compared to other mediums.

However, there was no omega-3 fatty acid detected in BBM medium for this study. Some technical errors might happen during the extraction off the BBM and 20% of CM even though the experiments have been repeated three times. 20% of CM (3.8% of C18:3n3) should have high omega-3 fatty acids compared to 30% of CM (9.1% of C18:3n3). Firstly, accessing the organic layer is a crucial factor to consider when choosing a lipid extraction method. Collecting the organic layer is problematic because non-lipid contaminants and salts can be transferred, lipid content might be lost in the process, and there is more space for analyst skill variability [21].

According to Karim *et al.,* the traditional lipid extraction method relies on the direct use of organic solvents to remove intracellular lipids, which is time-consuming and eco-friendly. Furthermore, various issues such as the rigid structure and composition of microalgal cell walls, the water content of biomass, the limited accessibility of lipids, reduced mass transfer, and the production of stable emulsions could all affect the extraction process [22].

3.3 Analysis of Astaxanthin

This research was continued by taking the astaxanthin content using a spectrophotometer. A previous study confirmed that the statistical analysis of first-order derivative spectrophotometry and HPLC revealed no significant differences. It was demonstrated that first-order derivative spectrophotometry is a quick and easy approach for determining astaxanthin in *H. pluvialis* that eliminates the unfavourable effects of astaxanthin with chlorophyll and beta-carotene [16]. Figure 2 shows the astaxanthin content in different mediums.

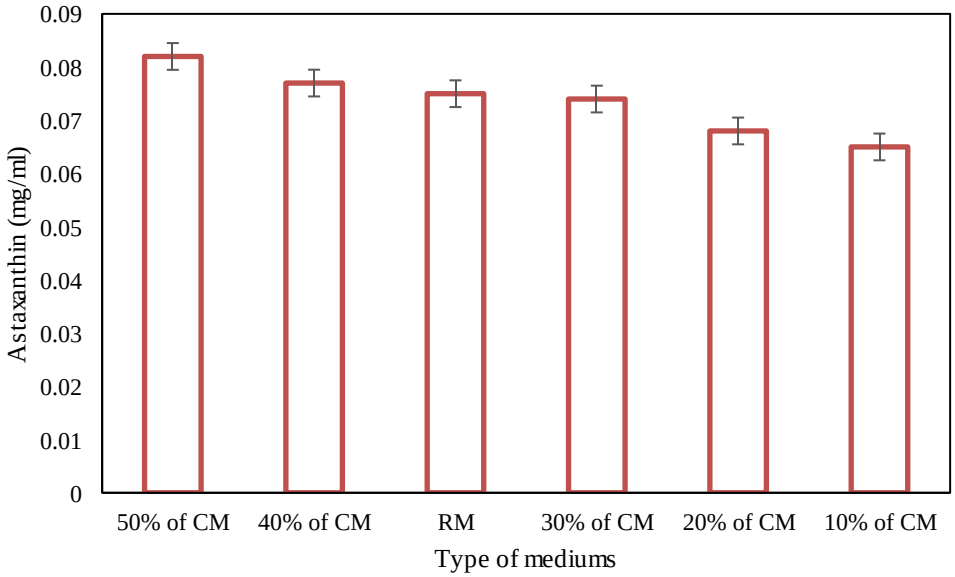

Fig. 2. The astaxanthin contain in *H. pluvialis* grown different type of mediums

Astaxanthin content was obtained from microalgae grown in the six media. The highest concentration was 50% of CM, followed by 40% of CM with 0.082 mg/mL and 0.077 mg/mL. Then, RM which is the control medium, has a concentration of astaxanthin of 0.075 mg/mL while 30% of CM has a concentration of 0.074 mg/mL. The lowest concentration content was obtained in 10% of CM (0.065 mg/mL) and was followed by 20% of CM (0.068 mg/mL).

According to Oslan *et al.,* phosphate is the most influential component for astaxanthin accumulation in *H. pluvialis* [23]. Since nutrients like phosphate are essential for microalgae metabolism, biomass growth in nutrient constrained media is lower than in nutrient replete media. Microalgae's photosynthetic ability is improved by the content of phosphorus, which promotes cell growth and proliferation. Furthermore, phosphorus is required for cell energy transmission, membrane growth, and nucleic acid and phospholipid manufacturing. As a result of the low availability of phosphorous in the growth media, cell growth would be hampered, and energy that would otherwise be used for cell division would be diverted to carotenogenesis [24].

There was limited study discussed regarding the exact amount of phosphate that is suitable for *H. pluvialis*. However, Figure 2 shows that 50% of CM and 40% of CM can accumulate a high amount of astaxanthin compared to other media. Hence, the amount of phosphate required for *H. pluvialis* growth in chicken manure medium is suitable for both concentrations.

4. Conclusions

The amounts of omega-3 fatty acids and astaxanthin produced in microalgae cultivated in commercial and chicken manure medium were determined at the end of this research. 10% of CM has the highest amount of omega-3 fatty acid compared to other mediums. This situation is due to the depletion of nitrogen in the medium, which causes the increase in fatty acid production in the microalgae. Astaxanthin production was influenced by the concentration of phosphorus in the medium. 50% of CM gave a high amount of astaxanthin production in *H. pluvialis*, which was 0.082 mg/mL.

A few improvements need to be made for future study. Firstly, the component and percentage composition in chicken manure medium needs to be investigated since this research focused on two main elements: nitrogen and phosphorus. Some of the nutrients in chicken manure may contribute to inhibiting the growth of microalgae. Hence, further studies regarding the nutrient's component in chicken manure medium and their effect towards the synthesis of omega-3 fatty acids and astaxanthin will need to be investigated.

Secondly, the other component of fatty acids obtained in *C. vulgaris* requires more research on how it can benefit the fish diet. Due to the Covid 19 pandemic situation, the usage of HPLC analysis to detect potential compounds in *H. pluvialis* was halted and cannot be repeated, thus the HPLC analysis for astaxanthin content in chicken manure medium is desired and can be carried out to further confirmed the compounds produced. Further research should include how to optimize astaxanthin and omega 3 using other growth parameters and statistical analysis using Taguchi Method can also be carried out, Comparison study of other potential animal wastes available in Malaysia can be done and this will support the circular economy and sustainable growth of microalgae by recycling nutrient from waste and at the same time producing various potential and beneficial bioproducts from microalgae.

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