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Comparison Growth Performance for H. pluvialis via an Automated Microalgal Nutrient Screening System against Flask Scale Cultivation

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ABSTRACT

Slow growth of Haematococcus pluvialis has prevented it from becoming a prominent competitor in production of natural astaxanthin. This is despite the fact that H. pluvialis is known to be an organism that is capable of producing high amount of astaxanthin in nature under stress conditions. In order to circumvent the slow growth H. pluvialis, new methods of improving the growth rate of H. pluvialis such as automated nutrient screening system has been used in the research. In this method, the screen was designed to optimize nutrients via 48 media formulation matrix for microalgae cultivation (150 µL microplate-based miniature-scale cultivation system). Improved cultivation media was formulated for H. pluvialis via the nutrient screening system. Medium formulation no. 6, 10, 43 and 47 (from the 48 media formulation) yielded the highest end-point OD_{560} of 0.926 ± 0.025, 0.840± 0.037, 0.839± 0.004 and 0.827± 0.006 respectively and all formulations were re-designated as medium HP1, HP2, HP3 and HP4 respectively for subsequent experiments. Reproducibility assessment of media (250mL flask scale experiments) was done to compare with miniature scale data. It is found that overall growth performance by laboratory scale is concordance with microtiter plate scale.

KEYWORDS: Automated Nutrient Screening System, Microalgae, Haematococcus Pluvialis, Nutrient, Media.

INTRODUCTION

Recently, a green microalga name H.pluvialis has gain popularity to produce strong anti-oxidant known as ketocarotenoidastaxanthin (3S-3′S-dihydroxy-β, β-carotene-4, 4′-dione) [12]. At present, astaxanthin is commercially available either from chemical synthesis or natural resources such as yeast, crustacean, cold-water fish by products and other microalgae [13]. Total market value of astaxanthin is over $240 Million per year and its selling price is around $2000 per kilogram in synthetic astaxanthin while natural astaxanthin is sold for over $7000 per kg [20]. Although chemical synthesis can provide a steady source of astaxanthin at large quantities, there are still concerns about its biological functions and food safety [31]. This is due to synthetic astaxanthin that typically contains a mixture of 3S, 3'S; 3R, 3'S and 3R, 3'R isoforms with a ratio of 1 : 2 : 1. In contrast, astaxanthin from microalgae is predominantly the 3S, 3'S isomer [10].

Among various astaxanthin produced by different species of microalgae, most of it have low than 1% of astaxanthin dry weight [10]. H.pluvialis however has been identified as the organism which can accumulate the highest level of astaxanthin in nature (1.5-3.0% dry weight) [20]. Due to these factors, astaxanthin produced from Haematococcus pluvialis cultivation has gained significant attention despite its low productivity yield (slow growth and low astaxanthin content), as a result of cultivation in non-optimal media [9].

There are various kind of microalgae species, even though it comes from similar algal group [21]. Each species nutrient consumption and environment can be varied from each other in order for them to grow optimally. Hence, the elements used and its composition may also be not similar correspond on its capability to adapt to the surrounding [7]. Carbon is one of the essential element as most of biological CO_2 fixation is required for photosynthesis of microalgae [17]. Nitrogen and phosphorus are macronutrients that are important for microalgae growth because they are involved in protein and nucleic acid synthesis as well as regulatory pathways in the cells [29]. Other elements such as calcium, magnesium, boron, iron, manganese, zinc, selenium, vanadium and silicon are also necessary for the balance growth of microalgae [30].

All these elements are fundamental for microalgae to maximize their growth rate. Thus, the right formulation and concentration is vital in order to obtain the best growth potential of microalgae. The automated nutrient screening system adopted in this research where to analyse improved production of element in N, P, Ca and Mg [36]. The aim of this paper is to determine the key nutrients to formulate new cultivation medium that can enhance growth and astaxanthin production of H. pluvialis and also to access its scability in a flask scale assessment.

MATERIAL AND METHOD

Microorganism and Culture Conditions

H. pluvilais (UTEX 2505) was obtained from the Culture Collection of Algae at the University of Texas, Austin, Texas, USA. This unicellular microalgae were cultivated using MES-volvox Medium which contained: 0.5 mM$Ca(NO_3)_2 \cdot 4H_2O$, 0.16 mM $MgSO_4 \cdot 7H_2O$, 0.16 mM Na_2glycerophosphate$\cdot 5H_2O$, 0.67 mM of KCl, 10 mM of MES, 0.5 mM of NH_4Cl, PIV Metal Solution which comprised of 2 mM of $Na_2EDTA \cdot 2H_2O$, 0.36 mM of $FeCl_3 \cdot 6H_2O$, 0.21 mM of $MnCl_2 \cdot 4H_2O$, 0.037 mM of $ZnCl_2$, 0.0084 mMof $CoCl_2 \cdot 6H_2O$, 0.017 mM of $Na_2MoO_4 \cdot 2H_2O$, followed by 1.02 x 10^{-5}mM of Biotin and 1.11 x 10^{-6}mM of Vitamin B_{12}. Temperature of culture was kept at 25°C while its agitation speed at 40 rpm [33]. pH of the culture was maintained at the range between 7.5 to 8.0 with 5%CO_2 enriched air for the aeration (0.2 VVM). For illumination, continuous light intensity of 40 ($\mu E\ m^{-2}\ s^{-1}$) was used [18].

Algae Growth Media Variation for Screening

The selection of element to be examined is based on comprehensive literature review. In the literature, a variety of medium used to cultivate H. pluvialis (NIES-C,N medium [19], MCM medium [32], BG11 medium [34], BBM medium [1], F1 medium [15], Kobayashi medium [28] and Basel medium [5]. Each of the medium was analysed for its elemental composition in order to identify the element that is frequently used. As the element found in the media can be variety, four main groups can be classified; namely nitrogen and phosphorus, macro-elements, micro-elements and other nutrients (vitamins, chelating agent and buffer).

Nitrogen and phosphorus can be considered to be a priority and must be initially optimised as without them, other element can be obscured. For example, algae that depends on ammonium to grow will have low growth rate in nitrate based. [27]. For the nitrogen sources, sodium nitrate ($NaNO_3$), urea (CH_4N_2O) and ammonium nitrate (NH_4NO_3) are the most regular chemical found in the media while for phosphorus sources is sodium hydrogen phosphate (Na_2HPO_4). The macro-elements (calcium and magnesium) were also included in the experiment as they play a major role in optimising the medium. The most common for calcium and magnesium sources based on extensive literature are calcium chloride ($CaCl_2$) and magnesium sulphate ($MgSO_4$) respectively.

Meanwhile for the micro-elements and other nutrients where are also essential to be included in the medium formulation, they are however added at constant concentration. The micronutrients were not optimized in this study because it was difficult due to its low concentration [4]. However, it is needed to ensure that the results are applicable for a larger scale such as photo-bioreactor and pond system [27]. Micro-elements used for this experiment consist of ferum (0.009 mM), cobalt (0.00005mM), copper (0.00005mM), chromate (0.0008 mM), manganese (0.005 mM), molybdate (0.0005 mM), Selenium (0.00003 mM), vitamin B_1 (0.0001 mM) and vitamin B_{12} (0.00001 mM). In addition, chelating agent (pH 8.0) and 10 mMTris-HCl (pH 7.5) are also included as buffer [9].

Automated Microalgal Nutrient Screening System (AMNSS)

The nutrient elements tested in the automated microalgal nutrient screening system consists of nitrogen, phosphorus, calcium and magnesium. All six common sources ($NaNO_3$, CH_4N_2O, NH_4NO_3, Na_2HPO_4, $CaCl_2$ and $MgSO_4$) have been chosen to be tested at 8 different concentrations. The range of concentration is also based on literature review in order to set the initial point and end point on where the probability the optimum might lie within. The sum of the samples is 48 and each of it was examined twice as a replicate that will make 96-well plate in total. Optical density at 560 nm wavelength (OD_{560}) were used as proxies for microalgal growth measurements. The algal cells were harvested by centrifugation at 4100 rpm for 10 minutes (NF 800, NÜVE Turkey). The cell pellets were washed once in 100 mMTris buffer before the nutrient screen experiment was carried out. Nutrient media was filter sterilized using 0.2 µm filter (Sartorius stedium, Germany) prior to nutrient screen experiments. Algal cells inoculation into sterile 96 microwell plates, dispensing of nutrient elements, CO_2 delivery and algal growth dynamics data collection were conducted as reported by [27]. The experiments were replicated 3 times for statistical purposes.

Analytical Method (Growth Rate Determination)

In order to trace the growth status of H. pluvialis, OD_{560} was used as the proxy for maximum specific growth rate measurements. The specific growth rate (μ) was calculated as follows:

$$\mu = \frac{(\ln OD_{560(t2)} - \ln OD_{560(t1)})}{t_2 - t_1}$$

The OD_{560} value of algal cell was monitored using a spectrophotometer (M1000 Tecan, Austria).

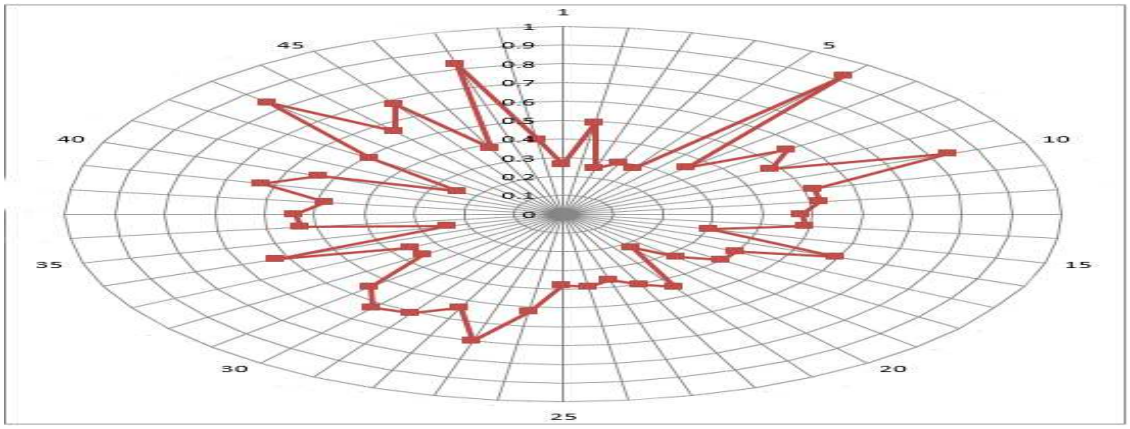
Reproducibility Assessment of H. pluvialis Growth Performance

Post screen data analysis, four media formulation with high end-point OD_{560} (referred as HP1, HP2, HP3 and HP4) were selected for reproducibility assessment trial using a small scale cultivation system (500 mL flask, cultivation volume 250 mL). HP medium was used as controlled medium for the experiment. Inoculums preparations were prepared as mentioned previously. The initial microalgal biomass density for each of the cultivation flasks was set at OD_{560} ~ 0.1. The cultures were continuously illuminated (dark: Light ratio of 24:0) at 40 µmoles $m^{-2}s^{-1}$ and were supplied with constant bubbles of CO_2: air mixture in a ratio of 1:99.

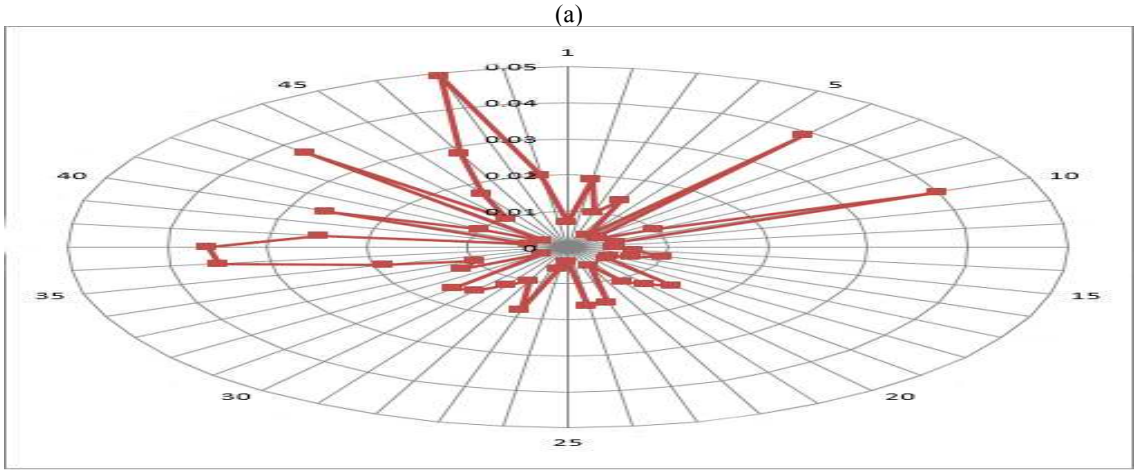
RESULTS AND DISCUSSION

Improvement of H. pluvialis Cultivation Medium via AMNSS

The automated nutrient screening [27] was designed to define strain specific media with non-limiting nutrients which can also minimize costs and to increase productivity of the microalgae. The automated nutrient screening system was conducted using 'matrix unit' which consists of 48 microwells variety concentration of nutrient in each well. The H. pluvialis cultivation process in the nutrient screening system was operated in photoautotrophic condition. As the formulation of nutrient used was different in each well plate, the growth rate and optical densities showed various results. The outcome has been plotted in the form of radial plot (end point OD_{560}) as shown in Figure 1(a).



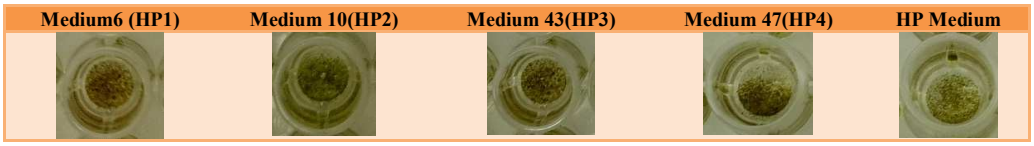
(a)



(b)

Number of Well	OD_{560}	Growth Rate (h^{-1})
6 (HP 1)	0.926± 0.025	0.039± 0.003
10 (HP 2)	0.840±0.037	0.040±0.014
43 (HP 3)	0.839 ±0.004	0.037 ±0.005
47 (HP 4)	0.827±0.012	0.049±0.021
HP medium	0.407±0.006	0.005±0.007

(c)



(d)

Figure 1: (a)Radial plot of the end-point OD_{560} of Haematococcus pluvialiscultivated in 48 media formulation solutions in the automated nutrient screening system, (b) Radial plot of growth rate of Haematococcus pluvialiscultivated in 48 media formulation solutions in the automated nutrient screening system, (c)Four (4) media formulations generating the highest end-point OD_{560} from the nutrient screening analysis, (d) Morphology and colour of H. pluvialis cells cultured in the microplate for media no 6, 10, 43, 47 and HP controlmedia

The radial plot shows that 4 different media (6, 10, 43 and 47) yielded high end-point of OD_{560}. The respective media formulation have been chosen to be investigated further to evaluate its potential by doing reproducibility assessment. In this research, three different types of nitrogen sources were used comprise of $NaNO_3$, $(NH_2)_2CO$ (urea) and NH_4NO_3. This is to identify which affinity of ion H. pluvialis tends to react (NO_3^- ,NH_4^+ or both) to achieve optimum growth rate. From the radial plot, formulation media number 43 and 47 showed amongst the highest end point OD_{560} (0.839±0.004 and 0.827±0.012) for nitrogen group where the concentration of NH_4NO_3 is 3.8 mM and 12.5 mM respectively. For other media formulations that involved nitrogen group most likely in the range of OD_{560} between 0.3 and 0.7. According to [2, 24], ammonia is known as the most effective nitrogen sources in terms of assimilation in microalgal cell while nitrate is the major form that exists in the environment. Thus, nitrate ion is vital in ensuring transportation and storage of nitrogen intake in the microalgal cell system. Besides that, most of nitrogen sources that used for media cultivation is usually in the form of ammonium salts or nitrates as they are readily available. In addition, low energy is needed for ammonium to be incorporated into cell [35].

Table 1: Comparative analysis of nnutrient formulation of improved HP1, HP2, HP3 and HP4 media with HP medium

Nutrient	HP Medium (mM)	HP1 (mM)	HP2 (mM)	HP3 (mM)	HP4 (mM)
KNO_3	4.055	4.055	4.055	-	-
NH_4NO_3	-	-	-	3.800*	12.500*
Na_2HPO_4	0.211	0.211	2.000*	0.211	0.211
$MgSO_4.7H_2O$	0.999	0.999	0.999	0.999	0.999
$CaCl_2.2H_2O$	0.748	2.000*	0.748	0.748	0.748
$FeSO_4.7H_2O$	0.009	0.009	0.009	0.009	0.009
$CoCl_2.6H_2O$	0.00005	0.00005	0.00005	0.00005	0.00005
$CuSO_4.5H_2O$	0.00005	0.00005	0.00005	0.00005	0.00005
Cr_2O_3	0.0008	0.0008	0.0008	0.0008	0.0008
$MnCl_2.4H_2O$	0.005	0.005	0.005	0.005	0.005
$Na_2MoO_4.2H_2O$	0.0005	0.0005	0.0005	0.0005	0.0005
Na_2SeO_3	0.00003	0.00003	0.00003	0.00003	0.00003
Biotin	0.0001	0.0001	0.0001	0.0001	0.0001
Tris-HCl, pH 7.4	0,0001	0,0001	0,0001	0,0001	0,0001
Vitamin B_1(thiamine hydrochloride)	0.00001	0.00001	0.00001	0.00001	0.00001
Vitamin B_{12} (Cyanocobalamin)	10.000	10.000	10.000	10.000	10.000

(*) Indicates the concentration difference of specific nutrients in TP control medium and improved HP1, HP2, HP3 and HP4 media.

For the phosphorus element (Na_2HPO_4), the radial plot number 10 showed OD_{560} value of 0.840±0.037 where its concentration is 2 mM. Phosphorus is essential in producing protein such as phospholipid, nucleotides and nucleic acid while maintaining the pathway for regulation of microalgal cell [26]. Besides that, inorganic phosphorus (mostly phosphate ion) has been reported to be used in significant excess as it is capable to form complex metal ion and helps in lowering bioavailability effects. Furthermore, the calcium element also showed positive results for media formulation number 6 by having OD_{560} at 0.926±0.025. Calcium is also an element that is necessary for uptake of microalgae. It is required for photosynthesis, macromolecules [22], ion transport [16] and as a secondary messenger of various responses to abiotic and biotic stimuli including light, high and low temperature [11]. Besides that, insufficient calcium consumption can cause reduction in number of daughter cells. [8]. Hence, metabolic regulation of calcium is crucial to avoid osmotic and metal stress which can lead to growth distortion of algal cells [25]. Even though magnesium does not show any high end point OD_{560} by which from the radial plot the value range is mostly between 0.2-0.5, it is an essential element to be present in the media formulation. Magnesium plays an important role which acts as central component of chlorophyll and is responsible to trap solar energy for photosynthesis [3]. In addition, it is also required for chloroplast structure

and helps in balancing equilibrium between grana and stromal region [14]. It is also reported that lack of magnesium can be a source of neutral lipid accumulation in microalgae[6].

Reproducibility Assessment of the Improved Media

Table 1 compares the nutrient concentration from the selected high OD_{560} during screening analysis. The difference between each medium (control, HP1, HP2, HP3 and HP4) lies in the concentration of specific element ($CaCl_2$, Na_2HPO_4 and NH_4NO_3) as labelled in Table 1. All of the media were cultivated in three replicates of 250ml culture in 500 ml flasks. The scale up difference for growth condition increase from a microwell plate screen (150 µL) into flask (250 ml), which is nearly 1500 fold volume with light path (6 fold) from 5~30 mm. Thus, certain parameters used during nutrient screening system might vary compared to cultivation using flask in regulating its optimal condition. Nonetheless, from the results (Figure 2), it shows that HP2 produce the highest end point OD_{560} with a value of 0.664±0.001.

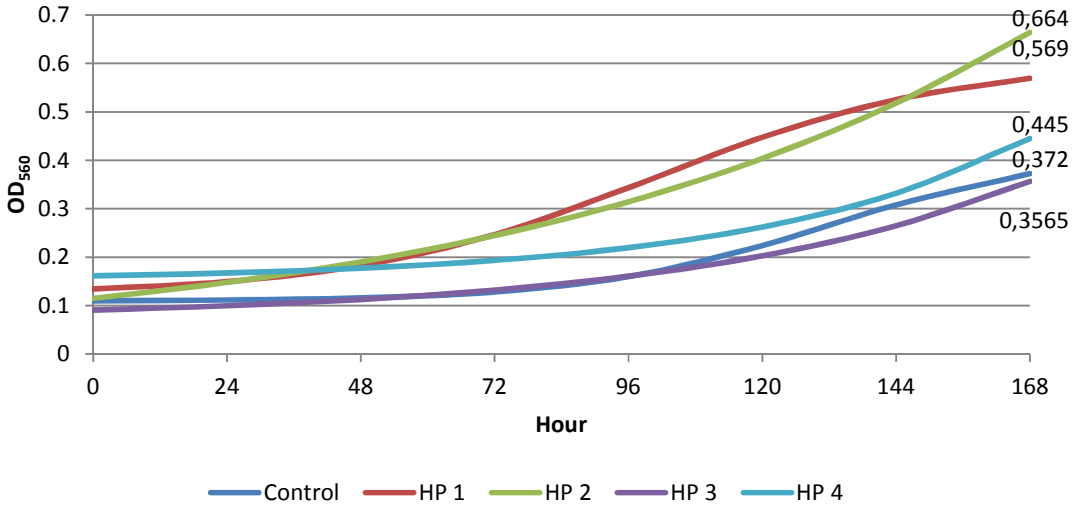


Figure 2: Graph of OD_{560} HP medium (control), HP1, HP2, HP3 and HP4 after 7 days

All of the medium were cultured in the flask with approximately similar starting point (OD_{560} ~ 0.1). From the graph in Figure 2, it can be recognized that each medium had various algal growth composition when are different in one avenue. Initially, day 1 and 2 showed gradual increment of OD_{560} values for all medium as lag phase begins. Starting day 3 onwards, all medium starts to grow exponentially until day 7.

Table 2: Comparison value of OD_{560} and growth rate of HP medium, HP1, HP2, HP3 and HP4 after 7 days in a miniature and flask scale

	Miniature Scale		Flask Scale	
Number of well	**OD_{560}**	**Growth rate (h^{-1})**	**OD_{560}**	**Growth rate (h^{-1})**
HP 1	0.926± 0.025	0.039± 0.003	0.569± 0.002	0.011± 0.003
HP 2	0.840±0.037	0.040±0.014	0.664±0.009	0.013±0.018
HP 3	0.839 ±0.004	0.037 ±0.005	0.356 ±0.013	0.006 ±0.007
HP 4	0.827±0.012	0.049±0.021	0.445±0.027	0.008±0.012
HP medium	0.407±0.006	0.005±0.007	0.372±0.001	0.006±0.005

From all OD_{560} values shown in Table 2, it can be concluded that the value is slightly lower in comparison to miniature scale as shown in figure 1(c). The highest growth rate achieve by HP 2 in flask scale is 0.013h^{-1}, which is lower than miniature scale 0.039 h^{-1}.

Several factors need to be taken into account when comparing miniature and flask scale cultivation. From the results, the difference in number of OD_{560} and growth rate value of both scale may come from light limitation factor. In miniature scale, it aids in prolonging the exponential phase due to sufficient light supply and mixing characteristics compared to flask scale which having light-limited linear growth after the exponential phase [23]. Furthermore, reduction of gas exchange rate may be involved as air to liquid surface area between microtiter plate and flask scale cultivation volume is reduced [36]. Hence, it is important to note that optimum nutrient formulation achieved in this study cannot be concluded as clear-cut for large scale cultivation. However, these results can contribute to be as a guideline towards larger scale trials in increasing H. pluvialis growth rate performances.

CONCLUSION AND RECOMMENDATIONS

The automated high-throughput nutrient screening is an efficient system in optimization of microalgae medium, which then enables growth rate to be increased. In future works, the concentration of the microelements can be further analysed to improve H. pluvialis growth performance. Besides that, reproducibility assessment can be upgraded to a larger scale such as photobioreactor to test its scalability.

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