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Development of Improved Formulation for *Neochloris oleoabundans* Via an Automated Microalgal Nutrient Screening System

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***Abstract*— Microalgal biomass has been regarded as one of the most promising commodities for biofuels production, fertilisers, animal food and high-value products. The capacity to generate high microalgal biomass is hence crucial to increase the number of the product that can be derived microalgal biomass. Recently, an advanced high-throughput nutrient screen was used to optimise the nutrient formulation to maximise the growth of *Neochloris oleoabundans* under photoautotrophic condition. The screen was designed to optimise 10 nutrients via the Box-Behnken experimental design that established a screen comprising 180 media formulations matrix for microalgae cultivation (150 µL microplate-based miniature-scale cultivation system). Improved cultivation media were formulated for *N. oleoabundans* via the nutrient screening system. Medium formulation no. 51 and 76 (from the 180 media formulation) yielded the highest end-point OD_{560} of 1.319 ±0. 009 and 1.345 ±0. 023 respectively and both formulations were re-designated as medium M1 and M2 respectively for subsequent experiments. Reproducibility assessment of both media (1 L larger scale experiments) was in concordance with miniature scale data. Nutrient main effect statistical analysis suggested that modulation of Ca, Mg, Fe, Mn and Zn concentrations may further improve the *N. oleoabundans* growth performance.**

***Keywords*— automated nutrient screening system; microalgae; *Neochloris oleoabundans*; nutrient; media**

I. INTRODUCTION

Microalgae are unicellular photosynthetic microorganisms that have shown its potential as one of the renewable sources [1] .Through the advancement of the technology, microalgae have been widely used as fertilisers, food sources, biofuels, and in pharmaceutical [2]–[4]. The advantages of microalgae as renewable sources are they have higher growth rate and productivity compared to agricultural crops and other plants. Microalgae also required less land area to culture compared to the other plants such as corns and rapeseed crops [5].

However, previous studies have shown that biomass productivity of microalgae generally is low and thus, more research needs to be conducted in order to attain high biomass production [6]. Therefore, the cultivation media that are being used need to be improvised in order to increase the biomass productivity and to find a medium that produces best growth rate. The nutrients that are being used in the media must satisfy the basic requirement for cell build up and metabolite production, by providing a sufficient supply of energy for biosynthesis and cell maintenance [7]. In the previous study, *Scenedesmus bijugatus* was cultured in different concentration of nitrogen to determine its biomass productivity. The experiment reported the highest biomass productivity of 0.63 g L^{-1}day-1 in 10 mM of sodium nitrate. This result was similar to *Neochloris oleoabundans* that were conducted by Li and co-researchers [8], [9]. Many studies have been conducted in order to increase the biomass productivity that usually involves the macronutrients. However, studies involving micronutrients are limited in number, although it may affect the growth of cells. From the previous work, the nutrients composition was determined by using stoichiometry equation. This equation was used to define medium composition to give maximum biomass concentration in terms of macronutrients specifically nitrates, phosphates and sulphates. The concentrations of micronutrients were not modified in this experiment. Nevertheless, it was reported, using stoichiometry does not guarantee an optimal composition since metabolite and micronutrients were not included in equation [10]. Other studied has reported micronutrients were difficult to optimise due to its low concentration used in the medium [11].

Microalgae vary greatly among their species, although they belong to a same algal group [12]. Their nutrient intakes and their growth conditions may not be similar for every species [13]. As the condition of growth for the microalgae could be varied, it is not surprising that their element composition varies. Carbon is reported to be an important element required for growth of microalgae [14].

The sources of carbon can be obtained from atmospheric

CO_2, flue gas and chemically fixed in the form of soluble carbonates such as $NaHCO_2$ and Na_2CO_3. However, atmospheric CO_2 levels are not enough to support the high growth rate of microalgae compared to flue gas [15]. Some microalgae species are able to grow with an organic compound such as sugars, molasses and acetic acid (heterotrophic or mixotrophic condition) [14]. Other than carbon, nitrogen is also important that required for microalgae growth [16]. Nitrogen is usually present in the form of nitrogenous compounds such as ammonia, nitrites, and nitrates [17]. Phosphorus is the third most important nutrient for microalgae growth. Phosphorus is needed for many cellular processes such as energy transfer and during the biosynthesis of nucleic acids [17]. Yet, Microelements such as calcium, magnesium, boron, iron, manganese, zinc, selenium, vanadium and silicon are also important for the growth of microalgae for the effective cultivations [16].

Each of these elements is vital and needed at different concentrations that can maximise the growth of various species of microalgae [18]. Therefore, the optimisation of nutrient in media is necessary to identify the best growth condition for microalgae. The high-throughput nutrient optimisation screen was used in this study where a large multi-dimensional statistical space matrix was used to identify improved production in terms of Ca, Mg, B, Fe, Cu, Mn, Zn, Se, V and Si [19]. The objective of this study was to optimisation the nutrients that required by *Neochloris oleoabundans* in order to obtain high biomass density. The Tris-Phosphate medium (TP medium) was reformulated using automated microalgal nutrient screening system [20].

II. Material and Method

A. Microalgae Strain Growth Medium and Inoculums Preparation

Neochloris oleoabundans was obtained from the microalgal library, University of Texas (UTEX), USA and maintained in the Microalgae Research Laboratory (MRL), Faculty of Applied Sciences, Universiti Teknologi MARA in TP solid medium under photoautotrophic condition (exposed to atmospheric CO_2). TP medium was modified from Tris-Acetate-Phosphate (TAP) medium [21] where the source of carbon, acetate was replaced with CO_2. TP medium formulation is indicated in Table 1. Microalgal cells inoculums were aseptically prepared by inoculating the algal cells from the solid medium into 100 mL of TP liquid medium and cultivated under the photoautotrophic condition and supplied with constant bubbles of CO_2: air in a ratio mixture of 1:99. The culture was continuously illuminated (light: dark ratio of 24:0) at 65 µmoles $m^{-2}s^{-1}$. Optical density at 560 nm wavelength (OD_{560}) and microalgal cell count was used as proxies for microalgal biomass density measurement. The algal cells were collected at log phase (OD_{560} ~ 0.8). 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 mM Tris buffer before the nutrient screen experiment was carried out.

B. Automated Microalgal Nutrient Screening System

The nutrient elements tested in the automated microalgal nutrient screen comprised of calcium, magnesium, boron, copper, manganese, zinc, selenium, vanadium, silicon and iron [20]. Specifically, the screen investigates three (3) concentration levels (+1, 0 and -1 representing high, medium and low concentrations, respectively) of all of the tested nutrient elements. Other nutrient elements and chemicals were supplied at a constant concentration as tabulated in Table 1. Formulations of the nutrient media and experimental design were conducted according to Radzun *et al.* [20] and Wolf, J. *et al.* [19] respectively. In this work, Box-Behnken statistical design was used to generate 180 nutrient media formulations matrix, and TP control medium replicates. Nutrient media was filter sterilised 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 Radzun *et al.* [20]. The experiments were replicated three (3) times for statistical purposes.

C. Reproducibility Assessment of N. oleoabundans Growth Performance

Post screen data analysis, two (2) media formulation with high end-point OD_{560} (designated as M1 and M2) were selected for reproducibility assessment trial using a larger – scale cultivation system (2 L flask, cultivation volume 1 L). TP medium was used as a controlled medium for the experiment. Inoculums preparation was 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 (light:dark ratio of 24:0) at 65 µmoles $m^{-2}s^{-1}$ and were supplied with constant bubbles of CO_2: air mixture in a ratio of 1:99.

D. Microalgal Cell Counting

Microalgal cell counting was conducted using a cell counting chamber (0.1 mm depth, 0.0025 mm2) (Neubauer, Germany).

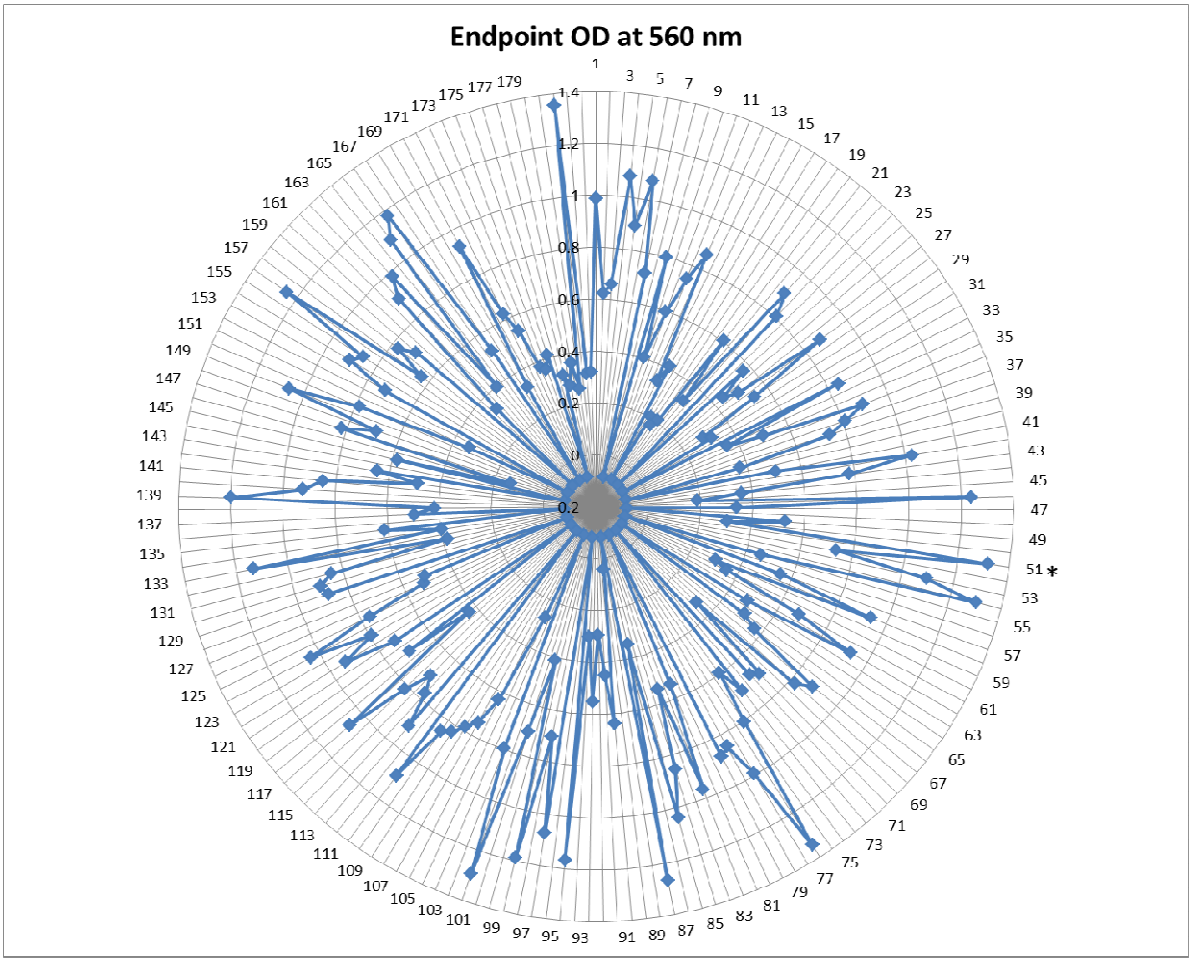
III. Results and Discussion

A. Improvement of N. oleoabundans Cultivation Medium Via Automated Nutrient Screening System

The automated nutrient screening [20] was designed to define strain specific media with non-limiting nutrients which can also minimise costs and to increase the productivity of the microalgae. The nutrient screening was operated using a screen matrix comprising of 183 microwells with different concentration of nutrients in each well (180 media solution and 3 replicates of TP control medium) [19]. The cultivation of the microalgae in the automated nutrient screening system was conducted under photoautotrophic condition. Fig. 1 – a shows the radial plot of the end-point $OD_{560.}$ Apparently, as the composition of micronutrients was varied in each microwell in the screen matrix, the growth and the final biomass densities in 180 wells were different. From the radial plot, there were several media that could be possibly produced based on its end-point OD_{560} of *N. oleoabundans* grown in the 180 media formulation. It was noted that 6 media formulations exhibited high end-point OD_{560} as tabulated in Fig. 1-b. Formulation of media

numbers 51 and 76 (end-point OD_{560} 1.319 ± 0.009 and 1.345± 0.023 and designated as medium M1 and M2 respectively) were chosen for further analysis in subsequent reproducibility assessment experiments.

The highest specific end-point OD_{560} values from the screening trial were subjected to main effects analysis using Minitab software (Fig. 2). This analysis involves the clustering of all conditions (for example, calcium) with low calcium (-1), medium calcium (0) and high calcium (1) concentrations. The average specific end-point OD_{560} and standard deviation of each of these -1, 0 and 1 clusters were calculated to determine whether they have a significant difference between them [20]. In this trial (Fig. 2), calcium was significantly preferable in high concentration (1) which was from 0.35 mM to 0.54 mM. High end-point OD_{560} was achieved when the higher concentration of calcium was used rather than in lower concentration (-1). A similar analysis was done to the other nutrients Zn, V, Mg, Fe, and Cu, where it can be seen that at high concentration (1), the high growth rate can be achieved in comparison to low concentration (-1). However, it is different for Si which required low concentration (-1) which was at 0.236 mM for better growth of the algal cells.



Number of well	OD_{560}
46	1.239 ± 0.027
51*	1.319 ± 0.009 *
54	1.302 ± 0.001
76*	1.345 ± 0.023 *
87	1.265 ± 0.052
102	1.293 ± 0.041
TP medium	0.3158 ± 0.002

(b)

Medium 51 (M1)	Medium 76 (M2)	TP medium
(a)		

(c)

Fig. 1 (a) Radial plot of the end-point OD_{560} of *Neochloris oleoabundans* cultivated in 180 media formulation solutions in the automated nutrient screening system. Asterisk indicates that medium 51 and 76 with highest end-point OD_{560} (b) Six (6) media formulations generating the highest end-point OD_{560} from the nutrient screening analysis (three (3) replicates. (c) Morphology and colour of *N. oleoabundans* cultured in the microplate for media no 51, 76 and TP control media.

With reference to main effect analysis (Fig. 2), calcium was preferred at high concentration for better growth of *N. oleoabundans.* Calcium plays an important role in photosynthesis of the microalgae. These include ion transport and as a secondary messenger of various responses to abiotic and biotic stimuli including light, high and low temperature [22]. Previous studies reported that calcium deficiency could cause the number of daughter cells to reduce and inhibition of formation of autospores [23].Thus, it is important to make sure the concentration of calcium is carefully regulated to prevent osmotic and metal stress which can lead to inhibition of algal cells [24]. Magnesium also was preferred at high concentration for the growth of microalgae. Magnesium is a major component of chlorophylls and a cofactor for enzymes [20]. Therefore, deficiency of magnesium can cause many metabolic disturbances to the culture, for example, metabolism of nitrogen can be disturbed, and it can be a temporary accumulation of carbohydrate materials. From the previous work, the growth of microalgae was tested in different concentration levels of magnesium. It was reported that at high concentration of magnesium, the cell division of microalgae were occurred rapidly compared to the low concentration of magnesium. However, it can be induced by adding more magnesium. It was also reported during the initial stage of cultural development with sufficient amount of magnesium, the rate of cell division was greater and small size of cells were present. As the culture developed, the magnesium became deficient, and cell division was stopped. During this stage, the cells were large but in the irregular form [25].

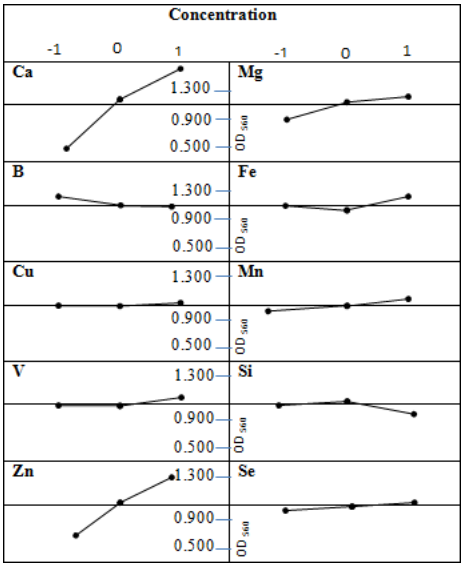


Fig. 2 Main and interaction effects analysis

B. Reproducibility Assessment of the Improved Media

Table 1 shows the comparative nutrient screening analysis for TP control medium and two improved media designated as medium M1 and M2 (previously designated as medium 51 and 76 respectively from the automated nutrient screen data). For media M1 and M2, the elements Ca, Mg, Fe, Cu, and Zn were supplied at the higher concentration as opposed to the TP medium. In addition, medium M1 and M2 differ in the concentration of Fe (higher in M2), Cu (higher in M2) and Si (higher in M1). The three media were used in the reproducibility assessment trials in three replicates of 1 L culture in 2 L flasks. The growth condition was scaled-up from a microwell plate screen (150 µL) to the flask scale (1 L) representing a condition approximately 6500 fold volume increase with 6-fold light path increased from 5~30 mm. Therefore, the optimal condition determined from the screen might be slightly different when scaling up as many parameters were involved. However, it was observed that the data of the large-scale cultivation of *N. oleoabundans* was in concordance with the miniature scale cultivation. It was observed that M2 medium yielded the highest end-point of cell density for *N. oleoabundans* followed by M1 and TP control media (Fig. 3).

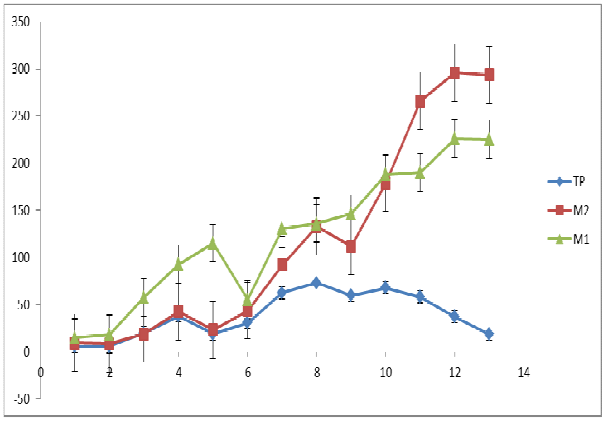


Fig. 3 Graph of cell density in TP control, M1, and M2 media

Both the improved and TP control media were cultured in the flask. The three media were at a similar starting point. After day 1, the cultures started to grow rapidly. However, each medium showed different algal growth since the composition of the media was varied. The growth of the algal cells in M2 was significantly higher than M1 and TP control medium. The cells density in M2 increased to 26.9 X 10^7 cells/mL and M1 was 22.5 X 10^6 cells/mL after 13 days. Cells productivity of M2 medium was higher than in TP control and M1 media which was 20.69 X 10^6 cells/mL/day while TP control medium was 1.48 X 10^6 cells/mL/day and M1 was 1.73 X 10^6 cells/mL/day, respectively.

The compositions of iron in these 3 media were also different (TP medium, 0.018mM; M1 medium, 0.0011 mM; M2 medium, 0.0020 mM). Iron is vital for the growth of microalgae, and it is required for the metabolic functions in photosynthetic electron transport, respiratory electron transport, nitrate and nitrogen reduction, sulphate reduction, dinitrogen (N2) fixation and detoxification of reactive oxygen species [26]. The concentration of iron in improved media was lower in comparison to TP control medium. The cellular requirement of iron varies with the nitrogen sources. High concentration of iron is required for the cells that grow in the nitrate compared with ammonium. However, a majority of the microalgae species used ammonium as the source of nitrogen because it is in a reduced form that is ready to be taken up by the algae cells. In contrast, nitrate assimilation requires energy for the reduction process (NADH usage and active transport via NO^{3-}) [19], [27].

TABLE I
COMPARATIVE NUTRIENT FORMULATION OF IMPROVED M1 AND M2 MEDIA WITH TP MEDIUM

Nutrient category	Nutrient	TP (Mm)	M1 (Mm)	M2 (Mm)
Nitrogen	NH_4Cl	4.12	4.12	4.12
Phosphate	KH_2PO_4	0.88	0.88	0.88
Macroelements	$CaCl_2$. 2 H_2O	0.35[a]	0.54[a]	0.54[a]
	$MgSO_4$. 7 H_2O	0.34[a]	0.79[a]	0.79[a]
Microelements	$Fe_2(SO_4)_3.7H_2O$	0.0018	0.0011[b]	0.0020[b]
	$CuSO_4.5H_2O$	0.0064[a]	0.0114[ab]	0.0180[ab]
	$MnCl_2.4H_2O$	0.0258	0.0258	0.0258
	$ZnSO_4.7H_2O$	0.077[a]	0.094[a]	0.094[a]
	H_2BO_3	0.184	0.184	0.184
	$(NH_4)_6MO_7O_{24}.4H_2O$	0.0009	0.0009	0.0009
	$CoCl_2.6H_2O$	0.0067	0.0067	0.0067
	Na_2SeO_3	0.0001	0.0001	0.0001
	$VOSO_4.H_2O$	0.000009	0.000009	0.000009
	Na_2SiO_3 . 5 H_2O	0.273	0.472[b]	0.150[b]
Chelating Agent	Na_2EDTA, pH 8.0	0.537	0.537	0.537
Buffer	Tris-HCl, pH 7.4	10	10	10
Vitamins	Vitamin B_1 (thiamine hydrochloride)	0.052	0.052	0.052
	Vitamin B_{12} (Cyanocobalamin)	0.0001	0.0001	0.0001

(a) The different of the nutrient concentration in TP control medium and the improved media, M1 and M2. (b) The different of nutrient concentration in M1 and M2 media

The concentration of silicon in M2 (0.236 mM) is lower than in M1 (0.472 mM). This element is usually present in the cell walls in many algal groups. It is an important element for diatoms especially for the growth, and basically, its cell wall is made of silica [28]. For green microalgae, silicon is not a major element required for growth. Therefore, silicon is not significantly required in M1 and M2. Cobalt is also required for the growth of the microalgae. It has a unique requirement in B_{12}, although the amount needed was small [26]. The concentration of cobalt in improved and TP control medium were 0.0067 mM. Previous studies have shown that the growth of microalgae increased due to the presence of high chlorophyll and carotenoids when cobalt concentration is in the range of 0.0084 mM to 0.0256 mM. When the concentration of cobalt is increased, the growth of microalgae is inhibited, and the cells have low pigment contents [29].

Other than that, copper also is an essential micronutrient for algal growth. Copper act as an enzymatic cofactor and electron carrier in the photosynthetic and respiratory processes in the medium [30]. The physiological function such as photosynthesis, mineral nutrient uptake, and water uptake was affected when the concentration of the metal compound such as copper was high. However, the concentration of copper in media M1 and M2 are still acceptable for the growth of the microalgae.

The concentration of zinc in M1 and M2 media was higher than control TP medium. Zinc can act as important enzyme co-factor for the microalgae growth, for example, in carbonic anhydrase, superoxide dismutase, and RNA polymerase. However, when the concentration of zinc was high in the culture medium, it can be toxic to the cells and cause degradation to the growth of the microalgae [31]. It was reported that the microalgae could tolerate zinc concentration range 0.26 mM to 0.07 mM. When the concentration was higher, the growth of the cells start to inhibit [32]

IV. CONCLUSIONS

The automated high-throughput nutrient screening allows rapid system optimisation of the microalgae medium which then enables the biomass density to be increased. After being optimized, the growth of microalgae was increased in improved media compared in control media (TP medium) from 1.48 X 10^6 cells/mL/day to 20.69 X 10^6 cells/mL/day. In future works, the concentration of the calcium, magnesium, iron, manganese, and zinc should be increased to gain higher biomass of the microalgae.

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