School of Engineering & Science
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Growth Modelling and Analysis of Microalgae Cultivation in Photo-Bioreactor

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Declaration

To the best of my knowledge and belief this thesis contains no material previously published by any other person except where due acknowledgment has been made.

This thesis contains no material, which has been accepted for the award of any other degree or diploma in any university.

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Abstract

A central composite design with 3-factor-5-level was applied to investigate the effect of environmental conditions and their interactions on the growth rate, biomass productivity and lipid productivity of *Chlorella vulgaris* in a 3L flat panel photo-bioreactor by varying environmental conditions: bicarbonate concentration, nitrate concentration and light irradiance. With applying simplex optimization, the average lipid productivity of 1.750 mg/L·day was obtained at the optimized process setting at 5 g/L bicarbonate concentration, 0.97 g/L nitrate concentration and light irradiance of 4500 LUX.

In this study, the growth rate and lipid productivity had been affected by interaction between bicarbonate and nitrate concentrations. As the cell population was increased by higher nitrogen element, higher carbon source demand was required for carbon fixation and cell growth. Another interaction demonstrated that the growth rate was enhanced by increases in nitrate concentration and light irradiance provided more light energy for high cell population to perform photosynthesis. On the other hand, another interaction showed that higher light irradiance provided sufficient energy to allow microalgae to assimilate more carbon element from higher bicarbonate concentration for cell growth and lipid production. However, further increase in bicarbonate concentration, nitrate concentration and light irradiance led to inhibition of cell growth and deterioration of lipid productivity. Hence, all interactions showed that the extent of the environmental condition strongly affected the effect of each environmental condition.

In conclusion, the present study has implication in the understanding of complexity of growth behaviour of *Chlorella vulgaris*, especially interaction from the combination of environmental conditions. With the understanding of growth behaviour of *Chlorella vulgaris*, the cultivation can be easily operated to meet the certain cultivation objective.

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Publications

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List of Abbreviations

1,3BPG – 1,3-Bisphosphoglycerate

ADP – Adenosine di-hydrogen phosphate

ATP – Adenosine tri-hydrogen phosphate

BBM – Bold's Basal Medium

CoA – Coenzyme A

CSCR – Curtin Sarawak Collaborative Research

CSIRO – Commonwealth Scientific and Industrial Research Organisation

EDTA – Ethylenediaminetetraacetic acid

G3P – Glyceraldehyde 3-phosphate

GOGAT – Glutamine oxoglutarate aminotransferase

MANOVA – Multivariate analysis of variance

$NADP^+$ – Nicotinamide adenine di-nucleotide phosphate ion

NADPH – Nicotinamide adenine di-nucleotide phosphate

PGA – 3-Phosphoglycerate

RuBP – Ribulose-1,5-bisphosphate

RuBPCO – Ribulose-1,5-bisphosphate carboxylase oxygenase

OFAT – One-factor-at-a-time

rcf – Relative centrifugal force

RSM – Response surface methodology

Chapter 1 Introduction

1.1 Background and Motivation

As human population increases and developing nations become more industrialized, the global energy demand increases continuously [37]. In this advanced technology era, only fossil fuel is capable of providing the huge amount of energy source to this world. Fossil fuel brought rapid development in human living environment and brought exponential growth in financial and industrial world since 1940. However, huge consumption on the natural and non-renewable fossil fuel for financial and industrial growth increases the demand on limited resource of fossil fuel. Since the remaining of fossil fuel becomes more difficult to find and access, it was reported that the fossil fuel reserve is depleting due to great demand of fossil fuel, [37]. Currently, the market price of crude oil is fluctuating around USD100 per barrel [67] and the inflation rate of the whole market in developing countries which are strongly dependant on crude oil might be affected.

At present, depletion of fossil fuel and the global warming became major global issue around the world. Searching for alternative, renewable and environmentally friendly resources of fuels becomes the only solution to solve the global issue of fossil fuel depletion. Alternative energy resources have engaged the attention of researchers for long especially with clean and renewable energy. Currently, alternative energy is produced from biomass, which can be processed into vegetable oils for biodiesel and sugar for bio-ethanol. Due to high food demand around the world, alternative energy from food source biomass is not encouraged.

On the other hand, scientists discovered that microalgae have great potential as non-food source for making biofuel. The intensive research of microalgae gave the world a major insight into the potential of microalgae as the next generation of alternative energy resources [44,60,70,80]. Microalgae are able to grow faster and contain higher oil content compared to traditional crops. The highest oil content can go up to 47% [29]. Besides that, it takes less space compared to traditional crops. As displayed in Figure 1-1, the annual biofuel production rate of microalgae is the highest among other traditional crops. This showed that microalgae are a strong competitive candidate in biofuel production compared to other traditional crop. In addition, microalgae are able to capture carbon dioxide from effluent to reduce global warming.

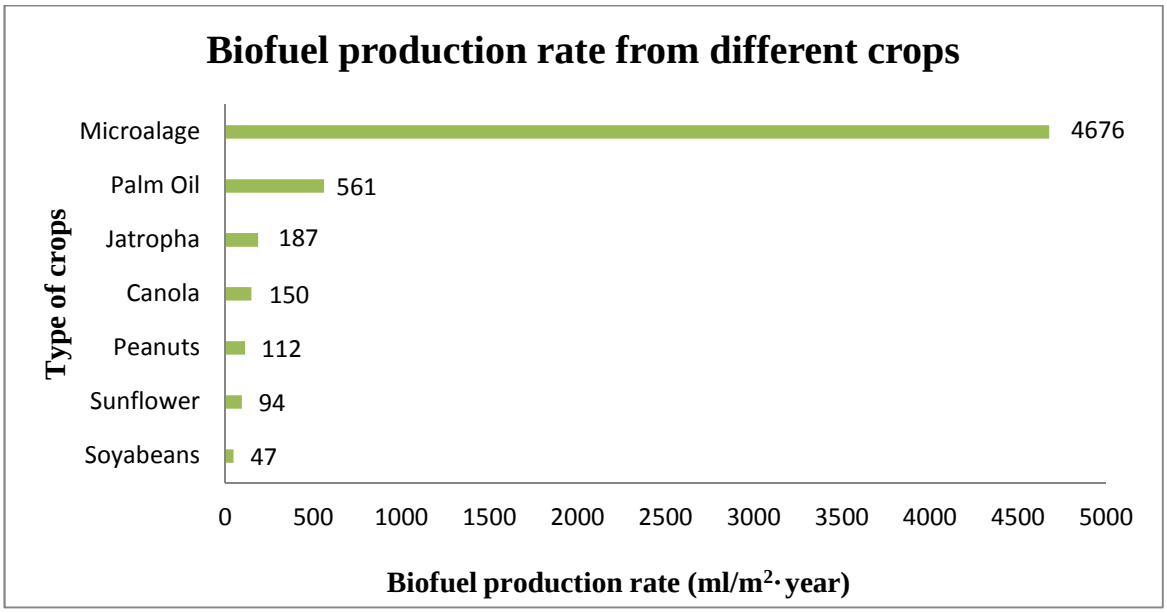

Figure 1-1: Biofuel production rate from different crops [93].

Unfortunately, the production of microalgae biofuel still faces a huge obstacle in mass production. High lipid production cost is the major obstacle limiting large scale microalgae production [83]. High lipid production cost is dependent on many factors, such as low yield of microalgae biomass, high cost of harvesting microalgae biomass and high cost of extracting lipid from microalgae biomass [99]. Taking high lipid production cost into consideration, the selling price of microalgae biofuel was set at

USD 32.81 per gallon [44]. This high selling price of microalgae biofuel still cannot compete with the low price of crude oil in the market (USD 100 per barrel / USD 3.174 per gallon [67]).

In order to make microalgae fuel economically feasible, microalgae cultivation must be improved so that the harvested bio-fuel is able to lower the production cost. This could be achieved through maximisation of the production rate of biomass and microalgae lipid. However, the manipulation of environmental condition controlling microalgae biomass and lipid production are not clearly understood, and neither is the relationship between environmental condition and microalgae productivity. In bio-process engineering, it is important to note that the understanding in microalgae growth is the central issue to be studied in this study. Therefore, maximising biomass and lipid production rate of selected microalgae by manipulating environmental conditions become motivation in this study.

It is found that previous researchers [13,57] applied One-Factor-At-a-Time (OFAT) method, which varies only one environmental condition and keep others at constant, investigating microalgae growth under different environmental conditions. Chen et al. (2008) investigated the growth rate, biomass productivity and lipid productivity of *Chlorella vulgaris* under the effect of potassium nitrate and sodium bicarbonate concentrations. On the other hand, Lv et al. (2010) investigated the lipid production rate of *Chlorella vulgaris* with varying light irradiance, carbon dioxide gaseous and potassium nitrate concentrations.

Both research studies performed by these scholars [13,57] provided the fundamental knowledge in the effect of individual environmental condition on microalgae cultivation. Although both studies presented that the highest growth rate, biomass productivity and lipid productivity of *Chlorella vulgaris* could be obtained under the effect of individual environmental conditions, the combinational effect between different environmental conditions (where two or more environmental conditions have effect upon one another) on *Chlorella vulgaris* growth was yet to be identified.

Because of the lack of research study in combinational effect between environmental conditions, the relationship between environmental conditions and microalgae growth is poorly understood. In order to identify the combinational effect between environmental conditions, statistical designed experiment is a more efficient approach to investigate two or more environmental conditions simultaneously in this study [18]. Therefore, response surface methodology was applied in this study because it is a simple and versatile tool in exploring the microalgae growth. Besides that, growth behavior of microalgae cultivation can be easily and effectively illustrated by mathematical modeling developed from response surface methodology. Besides that, with the developed mathematical modeling, the optimized experiment configuration can be obtained to maximize the outcome of microalgae cultivation.

In the research done by Xie et al. (2013), optimized experiment configuration for the highest oil production rate of *Chlorella sp.* was successfully obtained be performing optimization on developed mathematical model from central composite design illustrating oil production rate of *Chlorella sp.* under effect of glucose concentration, sodium nitrate concentration and temperature. Statistical analysis of MANOVA showed that combination effect between glucose and sodium nitrate concentration contributed effect on oil production rate of *Chlorella sp.* As increase in nitrate concentration increased the population of *Chlorella sp.*, high carbon source demand for oil production was counterbalanced by increasing glucose concentration.

In this study, the relationship of selected environmental conditions with microalgae growth of selected microalgae was clearly illustrated by mathematical modeling obtained from central composite design. With the obtained mathematical modeling, microalgae cultivation can be enhanced using optimized experiment configuration to produce maximum biomass and lipid production rate. Besides that, the optimized experiment configuration provided the fundamental process setting for the future work on selected microalgae.

1.2 Specific Aim and Objectives of Thesis

The overall aim of this research is to identify the contribution of different environmental conditions and their combinational effects on the growth of microalgae *Chlorella vulgaris* as well as to produce optimized cultivation conditions that give the optimum lipid productivity.

The specific research objectives are as follow:

- To identify individual and combinational effects of bicarbonate, nitrate concentration and light irradiance on growth rate, biomass productivity and lipid productivity of *Chlorella vulgaris*, by applying central composite design.

- To develop three models, which illustrate the growth rate, biomass productivity and lipid productivity of *Chlorella vulgaris* invidually.

- To determine optimal environmental conditions for lipid productivity with the aid of simplex optimization.

1.3 Thesis Structure

In this thesis, three mathematical models were developed to illustrate growth rate, biomass productivity and lipid productivity of *Chlorella vulgaris* under different effects of bicarbonate concentration, nitrate concentration and light irradiance. Additionally, lipid productivity of *Chlorella vulgaris* was optimized to obtain optimized process configuration for maximum lipid production rate.

As displayed in Figure 1-2, the methodology of this research was summarized for the model development and optimization. The development of this research did not follow a linear path but rather, if there was model mismatch and unexpected result, it will be revised until satisfied model and optimized process configuration were obtained.

However, the results presented in this thesis were the finalized experimental data without including model mismatch and unexpected result.

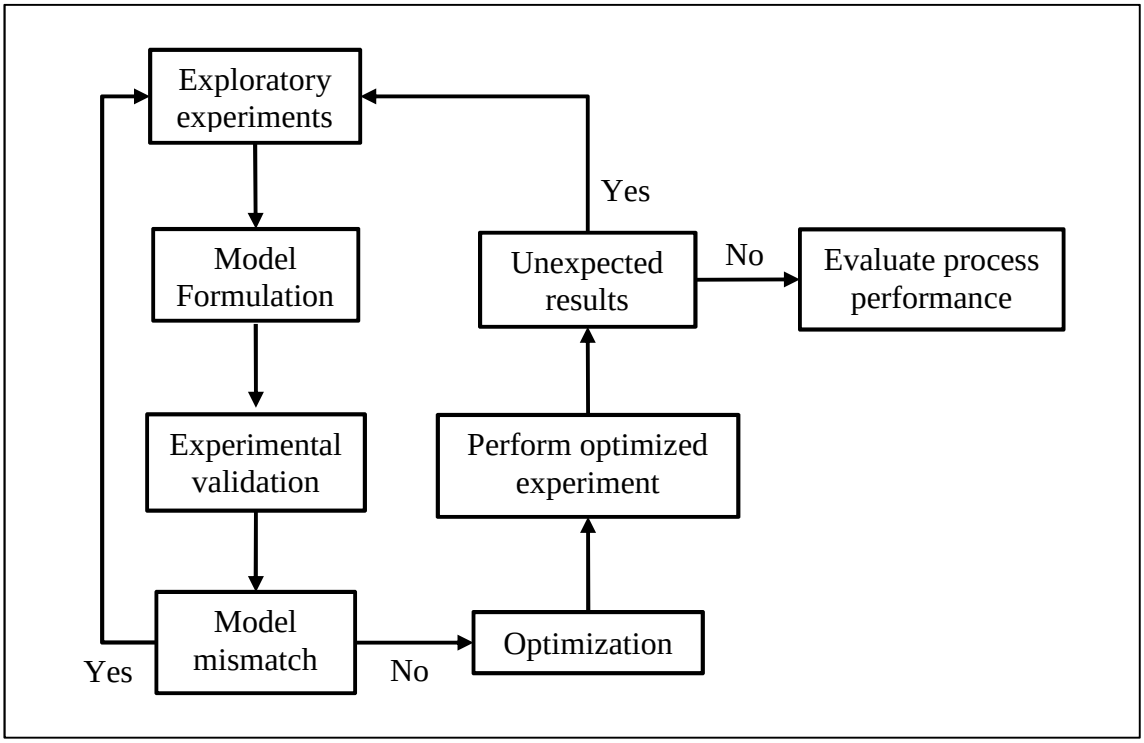

Figure 1-2: Flow diagram of the methodology followed for bio-process optimization.

This thesis is organised into seven chapters as outlined below.

- Chapter 1 defines the brief background, major scope and overall aim of this research.

- Chapter 2 reviews the current status of knowledge in the understanding of the contribution of each environmental condition in microalgae cultivation to different responses such as growth rate, biomass productivity and lipid productivity. This chapter also identifies the existing gap, from which the research objectives are developed.

- Chapter 3 summarises the research methodology, which consists of modelling approach and development, as well as experimental and analytical techniques employed in this study.

- The results and discussion are presented in Chapter 4. In Chapter 4, the effects of bicarbonate concentration, nitrate concentration and light irradiance on *Chlorella vulgaris* growth was discussed. This chapter was sectionalized into three sections which are growth rate, biomass productivity and lipid productivity of *Chlorella vulgaris*. Besides that, three different mathematical models were developed to illustrate the contribution of each model term on growth rate, biomass productivity and lipid productivity of *Chlorella vulgaris*.

- A mathematical model was developed to illustrate lipid productivity of *Chlorella vulgaris* under the effect of bicarbonate concentration, nitrate concentration and light irradiance in Chapter 4. In Chapter 5, the developed mathematical model was used to optimize lipid production rate of *Chlorella vulgaris*. The performance of optimization was discussed.

- Chapter 7 draws conclusions from this study and suggests the recommendation for the future development of the research.

Chapter 2 Literature Review

2.1 Introduction

Environmental conditions such as carbon dioxide concentration, water and light irradiance are essential factors in photosynthesis, which will significantly affect the growth of microalgae. The interactions between cultivation environmental conditions are also factors that must not be overlooked and require investigation as different combination of environmental conditions could have important impact on the growth rate and lipid yield of microalgae.

This chapter addresses the importance of the selected environmental conditions for this study, which are bicarbonate concentration, light energy and nitrate concentration, with the support of the literature review. Previous research outcome on the cultivation of microalgae under different growth conditions as well as modelling and optimization of lipid productivity are also presented. From the gathered evidence, suitable range of each selected environmental condition is determined for the experimental setup configuration.

2.2 Nature of Microalgae

Microalgae have been globally recognized as promising sustainable and environmentally friendly alternative source of lipid for biodiesel production. The characteristics of the microalgae, which have been widely reported are rapid growth, high biomass, high lipid content, sizing and high tolerance of extreme environmental condition and contaminant. Biomass productivity of the microalgae is higher than terrestrial crops [14] and the

microalgae can produce 30-100 times more energy per hectare compared to terrestrial crops [21]. Besides, atmospheric carbon dioxide or processed flue gas can be used as the source of the microalgae and salty or waste water can be used as culture medium for microalgae [82]. Unfortunately, high production cost makes the microalgae unfavourable in the biodiesel production. Therefore, more efforts are being put in the microalgae research.

Strain selection, which is the most difficult task, is being globally reported in literature [14,29,60,80]. The information, which was extracted from literature such as growth, biomass, lipid mass, size, costing, etc, shape up framework for strain selection. Recently, lipid productivity was reported as the key criteria in strain selection because lipid productivity consists of the data of growth rate, biomass and lipid mass [29]. Theoretically, lipid productivity is defined as the product of the biomass productivity and lipid content. Biomass productivity is the multiplication of growth rate and biomass. Higher biomass productivity would be desirable in order to reduce harvesting cost. On the other hand, higher lipid content is favourable in order to increase lipid productivity and to keep the cost of extraction as low as possible.

Chlorella, Spirulina and *Nannochloropsis* were proposed as potential candidate for the next biodiesel generation based on the growth, biomass productivity and lipid productivity [29]. Although *Spirulina* has high lipid productivity, *Spirulina* was reported that it consists of high protein, which is more suitable for human as food supplement [2]. Griffiths and Harisson (2009) showed that the *Nannochloropsis* has higher lipid productivity than *Chlorella*. The size of *Nannochloropsis* (about 2 μm) is smaller than *Chlorella*. However, during the harvesting process, the smaller size microalgae takes longer time to sink to the bottom when the sample undergoes centrifugation. This might increase the workload and expense of the harvesting process.

Chlorella is single celled and spherical green algae with the diameter of 2.0-10.0 μm. *Chlorella* can grow in both different habitats: fresh water and sea water. *Chlorella* strain was reviewed as one of the potential candidate for biodiesel production due to faster

growth and easier cultivation [70]. Furthermore, *Chlorella* is very hard to be contaminated by other species in open pond cultivation [76]. Illman et al. (2000) showed that the best growth rate of *Chlorella vulgaris* at 0.99 day^{-1}. The *Chlorella vulgaris* contains green photosynthesis pigments chlorophyll in chloroplast. Through photosynthesis, *Chlorella vulgaris* grows rapidly with only requiring three important crucial elements of carbon dioxide, water and light energy.

Dried chlorella generally consists of roughly 20% lipid [45] and Phukan et al. (2008) also reported that *Chlorella sp. MP-1* consists of high lipid content (28.82%) with low ash (5.93%). Rodolfi (2008) showed biomass productivity, lipid content and lipid productivity of *Chlorella vulgaris* were 0.17-0.20 g/L·day, 18.4-19.2% and 32.6-36.9 mg/L·day respectively under normal growth condition. Illman et al. (2000) showed that lipid content of *Chlorella* was increased from 18% to 40% during nitrogen depletion and lipid productivity was increased because of the increased lipid content. If nitrogen depletion was applied to the Rodolfi (2008)'s cultivation, the lipid content and lipid productivity would be expected as double of the value as mentioned before. Since *Chlorella vulgaris* is very easy to grow, has high growth rate and acceptable range of lipid yield, it was selected as the microalgae to be investigated in this study.

2.3 Photosynthesis

Photosynthesis is a metabolic pathway converting light energy into chemical energy to support other organism's biological activities. The process of photosynthesis is divided into two main processes: light dependent reaction and, light independent reaction or Calvin's cycle [77].

The initial step of light dependent process is the absorption of light energy by chlorophyll molecules in chloroplast. The absorbed light energy is utilized to produce oxygen gas, O_2 by reducing water molecule in the plant and drive the reduction of nicotinamide adenine di-nucleotide phosphate ion ($NADP^+$) into nicotinamide adenine di-nucleotide phosphate (NADPH), as displayed in the Equation 2-1.

$$2H_2O + 2NADP^+ \xrightarrow{light} 2H^+ + O_2 + 2NADPH \quad (2\text{-}1)$$

As displayed in Equation (2-1), the electron transfer is coupled between an electron donor (NADPH) and an electron acceptor (O_2) with the production of proton, H^+. This sets up electrochemical proton gradient generating chemical energy to allow the reduction of adenosine di-hydrogen phosphate (ADP) into adenosine tri-hydrogen phosphate (ATP), as shown in Equation (2-2). The addition of a phosphate group on protein molecules is defined as phosphorylation.

$$ADP + P_i + energy \rightarrow ATP \quad (2\text{-}2)$$

After the captured light energy is stored in the form of ATP and NAPDH in light dependent process, the major second process of photosynthesis is Calvin cycle or light independent process. The Calvin cycle occurs in three separate stages: carbon fixation, reduction, and regeneration of ribulose-1,5-bisphosphate, RuBP.

In carbon fixation phase, the carbon of carbon dioxide (CO_2), which is initially incorporated with 5 carbon compound of RuBP, is catalysed by the enzyme, ribulose-1,5-bisphosphate carboxylase oxygenase (RuBPCO) to produce unstable 6 carbon intermediate compound. Then, the intermediate compound is further decomposed into half to form two molecules of 3-phosphoglycerate (PGA) as final product of carboxylation.

In reduction phase, two molecules of PGA are initially converted into 1,3-bisphosphoglycerate (1,3BPG) by breaking a high energy phosphate group from ATP. Then, 1,3BPG is further reduced into glyceraldehyde 3-phosphate (G3P) by adding hydrogen bond from NAPDH.

When three CO_2 molecules are entering the Calvin's cycle, a total of six molecules of G3P are produced at the end of reduction phase. In order to keep Calvin's cycle looping, five out of six G3P molecules are regenerated to produce RuBP molecules by accepting

energy from three ATP molecules under enzyme. On the other hand, the remaining one molecule of G3P is discharged from the cycle to become a building block for the synthesis of large carbohydrates.

In order to produce triacylglyceride or lipid from glyceraldehyde 3-phosphate (G3P), lipid synthesis pathway of triacylglyceride involves with carboxylating acetyl coenzyme A (CoA), fatty acid biosynthesis, acyl group elongation and phosphate removal [15]. Energy derived from ATP was used to support catalytic activity throughout lipid synthesis pathway. Acetyl CoA carboxylase supported the carboxylation of acetyl CoA to produce malonyl CoA. In fatty acid biosynthesis, malonate was donated to acyl carrier protein from malonyl CoA with the support of malonyl CoA transacylase. Then, CoA from fatty acid biosynthesis was added with acyl group from fatty acid to form acyl CoA.

With glyceraldehyde 3-phosphate acyltransferase (GPAT) catalyst, acyl group was transferred from acyl CoA to G3P to form lysophosphatidic acid. Then, lysophosphatidic acid acyltransferase (LPAAT) catalyzes the transfer of another acyl group from acyl CoA to lysophosphatidic acid to produce phosphatide. After that, diacylgycerol was formed with the removal of phosphate group from phosphatide which was supported by phosphatide acid phosphatase (PAP) catalyst. Last step of lipid synthesis is to transfer an acyl group from acyl CoA to diacylglycerol to form triacyglyceride with diacylglycerol acyltransferase (DGAT) catalyst.

On the other hand, large quantity nitrogen element is also utilized for the synthesis of amino acid during photosynthesis process. Amino acid is served as the building blocks for the protein but also as starting points for the synthesis of many important cellular molecules including chlorophyll, chloroplast and nucleic acid [31,69,71]. In microalgae, nitrogen assimilation requires the reduction of nitrate to ammonium because nitrate or other nitrogenous compounds except ammonium, cannot be directly assimilated by microalgae [35]. One molecule of nitrate is reduced by nitrate and nitrite reductase to generate ammonium by consuming one molecule of NADH and six molecules of

reduced ferredoxin. Under glutamine synthetase catalyst, ammonium is assimilated via glutamine oxoglutarate aminotransferase (GOGAT) pathway leading to the production of glutamate. Glutamate is a molecule consists of carbon and nitrogen sources for the biosynthesis of amino acids. The operation of GOGAT pathway requires reducing power when nitrogen is presented in the highly oxidized form of nitrate.

As explained earlier, in light dependent process, light energy is absorbed and is stored into chemical energy in the form of ATP and RuBPH. While, these chemical energy will be reverted to ADP and RuBP to support nitrogen assimilation and light independent process converting CO_2 molecules into large organic compound. Hence, light energy, carbon and nitrogen sources are very crucial element in the photosynthesis.

However, huge change in environmental conditions could result in a significant loss in photosynthesis production [39]. The exposure of strong light energy could result in the degradation of chlorophyll and reduce the absorption and conversion of light energy in light dependent process [22]. On the other hand, weak light provide insufficient light energy for chlorophyll to produce chemical energy for light independent process or Calvin's cycle [26]. Three main phases of Calvin's cycle which are carbon fixation, reduction reactions and regeneration of RuBP, are gradually inhibited by strong or weak light irradiance, due to shortage of ATP and RuBPH from light dependent reaction. This phenomenon is identified as photo-inhibition.

When the microalgae grow under exponential phase, CO_2 is rapidly assimilated in Calvin's cycle with the energy supplied from ATP and RuBPH. However, low supply of CO_2 gas in the medium provided insufficient carbon source for microalgae to perform Calvin's cycle throughout microalgae cultivation. This explains why insufficient carbon source results in carbon depletion which inhibits carbon fixation phase of Calvin's cycle.

In order to supply sufficient CO_2 gas in medium for microalgae cultivation, researchers performed study on microalgae growth with variation of CO_2 gas supply in the range of 0% - 70% (v/v air) [89,90,106]. *Chlorella* is one of the most frequently used microalgae

for studies on the growth and high tolerance to CO_2 [4]. Research studies showed that increasing CO_2 concentration in medium enhanced growth rate, biomass productivity and lipid productivity of the microalgae cultivation. It was reported that maximum growth rate of *Chlorella vulgaris* was observed under 10% CO_2 gas supply [89,90,97,106]. However, the growth rate of *Chlorella vulgaris* was gradually decreased as the concentration CO_2 was further increased from 50% to 70%.

When ammonium production rate exceed nitrogen assimilation, the uncoupling of phosphorylation could be happened [17,49]. Consequently, further ammonium accumulation led to limitation of glutamine synthetase due to ATP deficiency. Besides that, glutamine production was gradually decreased because limited glutamine synthetase inhibited GOGAT pathway. Hence, growth was gradually inhibited due to GOGAT pathway inhibition.

In this study, microalgae population can be increased by increasing nitrogen element to increase the rate of microalgae producing lipid. However, lipid accumulation by high microalgae population increases the demand of carbon source and light irradiance in photosynthesis process. The effects of light irradiance, carbon and nitrogen elements were studied to develop mathematical model to illustrate microalgae growth. In order to achieve high lipid productivity of microalgae, optimization will be performed on mathematical model of lipid production rate in order to obtain optimized experiment configuration for this study.

2.4 Growth Dynamics

The growth dynamics of microalgae is generally characterized by five phases, which are lag phase, exponential phase, phase of declining growth, stationary phases and death phase. The growth dynamics of microalgae was presented in Figure 2-1.

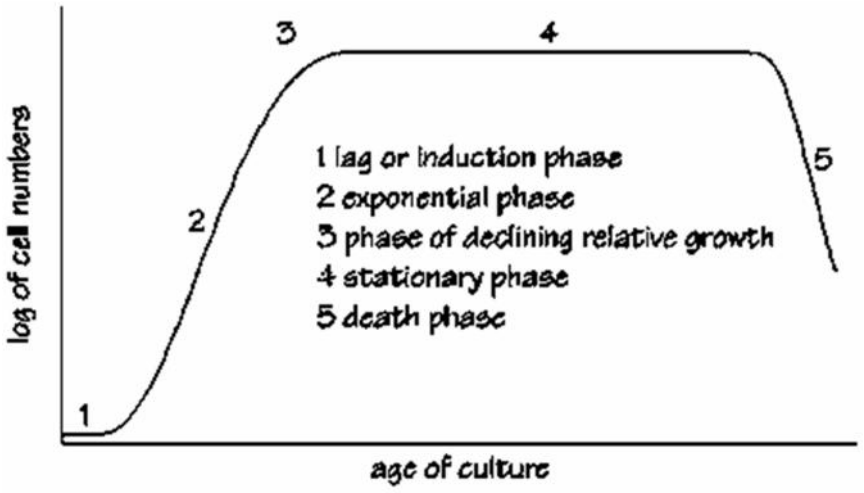

Figure 2-1: Five growth phases of micro-algae cultures [51].

When stock culture or inoculate is cultivated in the new culture medium, there will be notable changes in environmental conditions surrounding microalgae compared to previous cultivation condition [39]. The changes in environmental condition results in disturbing the balance between energy generated from light dependent process in chloroplast and energy utilized for light independent process. This is attributed to the physiological adaption of the microalgae metabolism in growth. Hence, there is very little increase in cell density. This period is named as lag phase. In order to effectively reduce the lag phase of upscale experiment, inoculated cultures with exponential growth is recommended to be cultivated in new cultivation.

In the exponential phase, carbon element is rapidly assimilated in medium with the chemical energy from light dependent process. When the microalgae grow into mature cell by storing the product from photosynthesis inside the cell, the mature size undergoes mitosis to divide into four daughter cells. The cell growth is greatly supported and cell density increases exponentially due to rapid cell division. Besides that, the biomass productivity and lipid productivity are also increased.

When the cultivation enters phase of declining growth rate (phase 3 in Figure 2-1), the cell growth gradually slows down because the physical or chemical factors gradually decreased or depleted.

In order to delay the phase of declining growth, the duration for exponential growth can be extended by introducing higher nitrate concentration into medium [43]. During photosynthesis, the nitrate is reduced into ammonia by a series of enzymes in microalgae [38] and the ammonia is then fused with carbon skeleton. This shows that the high consumption rate of nitrate in photosynthesis is similar to the rate of carbon assimilation during carbon fixation phase in light independent process [47].

During the stationary phase, constant cell density is resulted when the limiting factor and growth rate are balanced. The nutrient depletions, which are nitrogen depletion, iron depletion and etc will cause the lipid accumulation. The protein and carbohydrate are consumed by microalgae in order to survive during the harsh period of nutrient deficient. Although the cell growth is inhibited, the lipid composition is improved and lipid content is increased.

The final phase of growth dynamic is death phase where the cell density rapidly decreases and the culture eventually collapses. As limiting nutrient exhaust in culture medium, the microalgae growth is inhibited. In order to survive in the harsh period of nutrient exhaustion, microalgae consume organic compound stored inside microalgae. After the stored organic compound is consumed, microalgae starved to death.

In this research study, cell count was performed on daily basis to observe the growth dynamics. Microalgae will be harvested after they have reached stationary phase for three days because that indicates that the maximum growth has been achieved. On the other hand, growth rate of microalgae will be measured from phase 2 to phase 3 as the calculation was made based on the exponential growth. Formula used for growth rate calculation is presented in Section 3.8.

2.5 Effect of Different Environmental Conditions

In cultivation of microalgae, various environmental conditions affecting microalgae growth has been studied by previous researchers, in particular initial culture pH, pH maintenance, temperature, medium composition and light irradiance.

One of the important operational conditions affecting growth behaviour is medium composition, especially carbon and nitrogen sources as they are used to produce organic compounds. Various organic carbon compounds, which are acetate, glucose and glycerol, can be served as sole carbon source for carbon fixation phase in *Chlorella vulgaris* [54]. Combinational effect of glycerol and glucose produced higher growth rate, biomass mass and biomass productivity compared to glycerol as sole carbon source [48]. On the other hand, the research study by Jin et al. (2006) demonstrated that the exponential phase of *Chlorella vulgaris* growth can be extended by introducing nitrogen element. The effect of various nitrogen compounds, which are ammonia, nitrate and urea on microalgae growth have been studied [24,32-34,94].

Apart from carbon and nitrogen compounds, light is also considered as crucial element in productivity and yield of photosynthetic reactions. Light energy is collected by chlorophyll and then is converted into chemical energy to fuel light independent process where carbon elements are converted into large organic compound. As microalgae growth is enhanced by supporting nutrient, cell population is increased and stronger light irradiance is required for microalgae to perform light dependent process. Besides that, high lipid yield was reported under continuous illumination of high light irradiance [28]. However, the microalgae growth would be inhibited if microalgae are exposed under excessive light irradiance.

Initial culture pH also brought impact on growth rate [61,96] and lipid production [5,96] of *Chlorella vulgaris*. Mayo (1997) studied the growth rate of *Chlorella vulgaris* over the pH range of 3.0 - 11.5. Similar finding was presented by Yeh et al. (2010) that *Chlorella vulgaris* was also not significantly inhibited over the range of pH 5 - 10. *Chlorella vulgaris* had the ability to tolerate at pH value of 3.0 and 11.5 [61]. However,

the maximum growth rate of *Chlorella vulgaris* is observed at pH value of 7.0. As the pH value further decreased to pH 3.0 and increased to pH 11.5, the growth rate of *Chlorella vulgaris* was gradually decreasing. On the other hand, the maximum biomass and lipid productivity of *Chlorella sp* and *Tetraselmis suecica* was found at pH 7.5 and pH 7.0 respectively [63]. The optimum pH range for enhancing the growth rate and lipid production of *Chlorella vulgaris* was at pH 6.5 – 7.0 and 7.0 – 8.5 respectively [96].

In experimental work performed by Chen et al. (2010) and Yeh et al. (2010), bicarbonate salt in culture medium was assimilated by *Chlorella vulgaris* as carbon source and high lipid was accumulated within *Chlorella vulgaris* during photosynthesis. Introducing bicarbonate into culture medium creates alkaline buffer solution at pH 8.0 which can resist large change in pH by adding acid or alkali into solution. However, adjusting medium pH by adding acid or alkali would change the nature of bicarbonate in medium. When the bicarbonate reacts with alkali, bicarbonate is decomposed into carbonate salt and water. On the other hand, bicarbonate reacts with acid to produce salt and carbonic acid which is readily decomposed into water and CO_2 aqueous. As the solubility of carbon dioxide gas in water is low, carbon dioxide aqueous will shift equilibrium into carbon dioxide gaseous and escape from solution [85]. This will allow CO_2 gas escaping from culture medium into atmosphere and carbon source concentration will be decreased in culture medium. Due to the loss of bicarbonate as CO_2 gas, the effect of carbon source on microalgae growth is unable to be clearly investigated with pH regulation by adding acid into culture medium. Besides that, alkaline buffer solution of pH 8.0 fall within the acceptable optimum pH range for lipid production [96]. Hence, pH regulation by adding acid or alkali is not necessary to be applied in the culture medium containing bicarbonate.

It was also found that temperature could affect the microalgae growth. Mayo (1997) studied kinetic growth of *Chlorella vulgaris* by varying medium temperature between 10°C and 40°C [61]. This study showed that maximum growth rate of 0.5 day^{-1} for *Chlorella vulgaris* was observed at optimum temperature of 32.4°C. The growth was

gradually inhibited when the pH value is approaching to boundary limit from optimum temperature. In this study, medium temperature was maintained at 30°C.

As mentioned above, microalgae cultivation is significantly affected by various environmental conditions. However, it is very challenging and costly to manipulate all environmental conditions within desired range at outdoor large scale microalgae cultivation. Amongst different environmental conditions, it is found that it is more reasonable, economical and feasible to control medium composition and light intensity, if compared to other parameters in improving the growth of algae.

Besides, medium compositions and light intensity were found to have greater impact on the growth of microalgae relatively. Nitrogen element can efficiently increase population of selected microalgae. As microalgae population is increased, the demand of light energy and carbon source are increased for microalgae producing lipid. Stronger light irradiance is needed for high microalgae population to convert light energy into chemical energy in light dependant process. With sufficient chemical energy derived from light dependant process, higher carbon source concentration is required to process carbon element into large organic molecule stored within microalgae. Hence, carbon element, nitrogen element and light irradiance are the three selected environmental conditions to be studied in this research. In the following section, previous research performed on selected environmental conditions will be presented in detail.

2.5.1 Effect of Bicarbonate in Algae Cultivation

During photosynthesis of the plants, carbon dioxide gas, $CO_{2(g)}$ and water are converted into oxygen gas and organic compounds (refer to Equation 2-1), especially sugar in the presence of chlorophyll and light energy. In fact, the $CO_{2(g)}$ concentration in culture medium is one of the crucial factors influencing algae growth.

$$CO_2 + 6H_2O \rightarrow C_6H_{12}O_6 + 6O_2 \quad (2\text{-}1)$$

In this respect, a lot of attention was given to investigate the growth of microalgae under different $CO_{2(g)}$ concentration in air flow. Researchers found out that most *Chlorella vulgaris* strains are able to demonstrate excellent tolerances to high concentrations of $CO_{2(g)}$ up to 70% [89,90,106]. Thus, several kinetic growth studies of *Chlorella vulgaris* were explored under different ratio of $CO_{2(g)}$ in the range of 0% - 70% (v/v air). When *Chlorella pyrenoidosa* was grown under high concentration of carbon dioxide gaseous, high photosynthesis efficiency was observed at low oxygen concentration in medium [86]. However, as cultivation was continued to cultivate under high concentration of carbon dioxide gaseous, oxygen concentration in medium was increasing and cell had difficulty to export oxygen from cell to medium. It was found out that *Chlorella vulgaris* exhibited the maximum growth rate at 10% carbon dioxide [89,90,97,106], high growth rate and cell concentration were still observed between 30% and 50% $CO_{2(g)}$. With the increment of $CO_{2(g)}$ concentration from 50% to 70%, the growth rate became very sluggish. However, the duration of lag phase was lengthened when the $CO_{2(g)}$ concentration was increased from 10% to 70%.

Sorensen *et al.* (1996) and Shakhashiri (2008) explained the general chemistry of $CO_{2(g)}$ dissolving in water. As $CO_{2(g)}$ was pumped into culture medium, partial of $CO_{2(g)}$ was dissolved into culture medium to form $CO_{2(aq)}$ because the solubility of $CO_{2(g)}$ in water is about 900cm^3 CO_2 per 1L water.

$$CO_{2(g)} \rightarrow CO_{2(aq)} \quad (2\text{-}2)$$

Then, a chemical equilibrium (2-3) is established between carbonic acid, H_2CO_3 and $CO_{2(aq)}$.

$$CO_{2(aq)} + H_2O_{(l)} \leftrightarrow H_2CO_{3(aq)} \quad (2\text{-}3)$$

Carbonic acid, H_2CO_3 is a weak acid, which will be easily dissociated into bicarbonate, HCO_3^- and carbonate CO_3^{2-} ions.

$$H_2CO_3 \leftrightarrow H^+ + HCO_3^- \quad (2\text{-}4)$$

$$HCO_3^- \leftrightarrow H^+ + CO_3^{2-} \quad (2\text{-}5)$$

When the $CO_{2(g)}$ supply is running low through the culture medium, $CO_{2(aq)}$ will be depleted because algae continuously assimilate $CO_{2(aq)}$ for photosynthesis process. If carbonate CO_3^{2-} ions are introduced into culture medium, the chemical equilibrium (2-4) shifts to build more bicarbonate ions, HCO_3^-, which extract hydrogen ions, H^+ and thus increase the pH of the medium. Additional of bicarbonate ion, HCO_3^- also shifts the chemical equilibrium (2-5) to form carbonic acid, H_2CO_3 and brings the medium to higher pH value. In order to compensate the reducing $CO_{2(aq)}$, the carbonic acid, H_2CO_3 decompose and form water and $CO_{2(aq)}$.

Riebesell *et al.* (2000) reported that the increment of $CO_{2(aq)}$ concentration dramatically increased the biomass. However, due to the issue of solubility of $CO_{2(g)}$ in water, further increase in $CO_{2(aq)}$ concentration by increasing the flow of $CO_{2(g)}$ is unfavourable. Wijanarko et al. (2008) studied the concentration of bicarbonate ion formed in the culture medium by supplying 10% CO_2 gas. The obtained maximum bicarbonate concentration was 2.94mM in single reactor and average bicarbonate concentration was 3.39mM in multiple series of reactor. Hence, adding carbonate ion, CO_3^2 [47] or bicarbonate ion, HCO_3^- [42,72,78,79,87,102,103,105] into solution become alternative option to boost the $CO_{2(aq)}$ concentration in culture medium. From the view point of economic feasibility, sodium bicarbonate is a cheap carbon source compared to carbon dioxide cylinder tank because sodium bicarbonate can be easily manufactured through chemical adsorption, which can effectively remove carbon dioxide gaseous with sodium hydroxide aqueous from industrial effluents [37,62,76].

Jeong et al. (2003) showed that the highest growth of *Chlorella sorokiniana* was achieved at 152.83 µM sodium bicarbonate under 20% CO_2 gas supply and temperature of 40°C. Richmond et al. (1982) studied the highest growth rate of *Chlorella vulgaris* ($3.83x10^{-3}$ day^{-1}) under 4.2 g/L, 8.4 g/L and 16.8 g/L $NaHCO_3$ without CO_2 gas supply and found out that *Chlorella vulgaris* grew well at 4.2 g/L bicarbonate. Further increment of 4.2 g/L bicarbonate will increase the inhibitory effect on the growth on *Chlorella vulgaris*. Chen et al. (2010) studied the change of the growth behaviour of

Chlorella vulgaris between 0.1 g/L and 2.0 g/L sodium bicarbonate in Figure 2-2. It is observed in Figure 2-2 that the growth rate, biomass productivity and lipid productivity increased with increasing sodium bicarbonate concentration but they slightly decreased after 1.5 g/L sodium bicarbonate. On the other hand, the lipid content decreased as the sodium bicarbonate concentration increased.

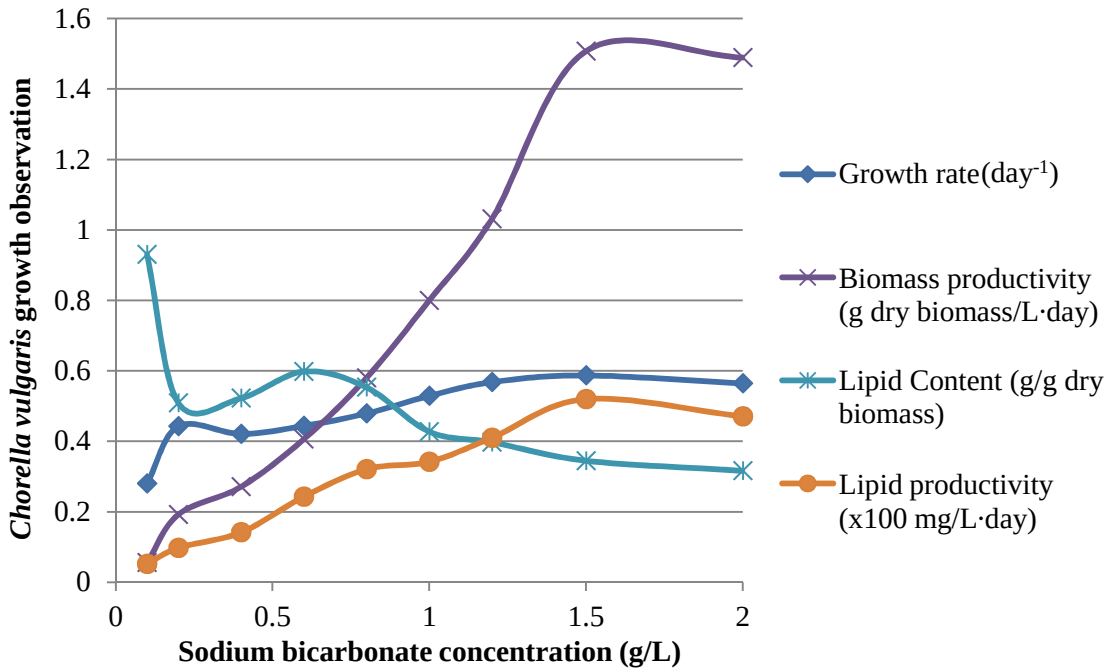

Figure 2-2: Growth rate, biomass productivity, lipid content and lipid productivity of *Chlorella vulgaris* with varied sodium bicarbonate concentration (g/L) [13].

The research performed by Yeh et al. (2010) showed the optimal environmental conditions (3072 LUX and 1g/L $NaHCO_3$) for the cultivation of *Chlorella vulgaris* based on the carbon source utilization. With optimal bicarbonate concentration of 1 g/L, the biomass productivity (113 mg/L·day), lipid content (35%) and lipid productivity (39.55 mg/L·day) of *Chlorella vulgaris* were obtained in Yeh et al. (2010)'s study. As mentioned earlier, at 0.65 g/L nitrate concentration, Chen et al. (2010) produced the highest growth rate, biomass productivity and lipid productivity which were 0.587 day^{-1}, 115.7 mg/L·day and 60.5 mg/L·day respectively at 1.5 g/L bicarbonate concentration. However, the highest lipid content of 9.3% was observed at 0.1 g/L sodium bicarbonate.

On the other hand, Widjaja et al. (2009) showed biomass productivity (36.50 mg/L·day), lipid content (26.71%) and lipid productivity (9.75 mg/L·day) under the flow rate of 20 ml/min carbon dioxide gas and 6 L/min air. The lipid content and lipid productivity of Yeh et al. (2010) and Chen et al. (2010) were higher than Widjaja (2009) showing that microalgae grown in bicarbonate could possibly perform better than those grown in culture with carbon dioxide gas. Furthermore, lipid content of 35% from Yeh et al. was much higher than regular reported value of 25% - 30% [45,70,101]. This experiment strongly supported that $NaHCO_3$ is able to improve the biomass productivity and lipid productivity during cultivation.

Besides that, the relative rate of photosynthesis of *Chlorella vulgaris* in $KHCO_3$ solution is higher than $NaHCO_3$ [73]. At the first few hours of cultivation, the rate obviously increased in the 10 g/L $KHCO_3$ but decreased in the 10 g/L $NaHCO_3$. This is mainly because although $KHCO_3$ and $NaHCO_3$ have similar chemical and physical property, they play different role in photosynthesis metabolism. Generally, sodium is not considered as essential element in photosynthesis. On the other hand, potassium is definitely essential element for plant metabolism that is involved in photosynthesis because potassium is used to control the opening and closing of the stomata of the plant for water regulation in the plant.

As discussed above, Yeh et al. (2010)'s experiment clearly showed that bicarbonate was able to boost biomass productivity and lipid productivity of *Chlorella vulgaris* compared to Widjaja et al. (2009). Pratt et al. (1940) also concluded that the relative rate of photosynthesis of *Chlorella vulgaris* was higher in $KHCO_3$. Therefore, $KHCO_3$ was selected to be studied in this research. As Richmond et al. (1982) and Yeh et al. (2010) commented that applying below 10 g/L bicarbonate concentration was better in terms of growth, biomass productivity and lipid productivity, the range of $KHCO_3$ concentration to be used in this study is between 2.5g/L and 7.5g/L.

2.5.2 Effect of Nitrate in Algae Cultivation

Nitrogen is the most significant element in the nutrient to regulate the growth of *Chlorella vulgaris* in cultivation provided that there is adequate photonic energy [56,59,94]. Besides that, the exponential phase of microalgae growth can be extended by introducing higher concentration of nitrogen element [43]. It is also reported that nitrogen is found in the porphyrin molecule, which is found in the chlorophyll structure and cytochrome enzymes [55,100]. On the other hand, nitrogen is important in the photosynthesis and in the protein synthesis, which involves the synthesis of purines and pyrimidines of RNA and DNA.

It was reported that *Chlorella* strains are able to assimilate various nitrogenous compounds [6,24,32-34,52,94] and the rate of assimilation of different nitrogenous compounds by *Chlorella elliposoidea* was sorted out in the order of rapidity: ammonia > urea > nitrate [32]. Although nitrate was the slowest in the order, nitrate still produced the highest lipid content and lipid productivity compared to urea and ammonia [52].

The increment of ammonia will raise the pH of culture medium, which will inhibit the growth of algae in culture medium [17,49]. On the other hand, it was found that addition of urea [25,36] and nitrate [16,53,65,94,101] dramatically increased the growth, biomass and lipid productivity. In other words, algae bloom is expected from excess of nitrogenous compound. However, despite that high amount of nitrogenous compound definitely increased the cell growth, biomass productivity and lipid productivity were found to decrease [29,40,52,53,84,101]. This research evidence was strongly supported by Li et al. (2008) and Chen et al. (2010)'s experiment regarding the change of growth behaviour of different microalgae under variation of nitrate concentration.

Chen et al. (2010) investigated the growth behaviour on *Chlorella vulgaris* by varying potassium nitrate concentration between 0 g/L and 2.6 g/L in Figure 2-3.

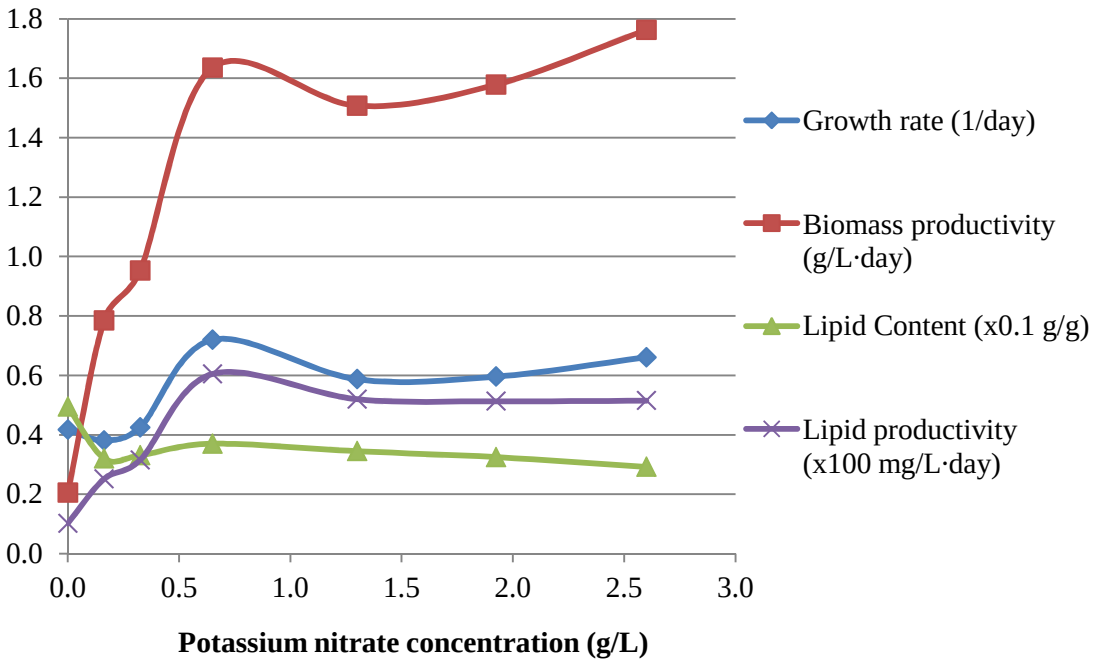

Figure 2-3: Growth rate, biomass productivity, lipid content and lipid productivity of *Chlorella vulgaris* by varying potassium nitrate concentration (g/L) [13].

It is noticed that the increasing nitrate concentration (0 g/L to 0.65 g/L) dramatically increased the growth rate, biomass productivity and lipid productivity of *Chlorella vulgaris*. However, the growth rate, biomass productivity and lipid productivity slightly decreased at 1.3 g/L nitrate concentration. When the nitrate concentration was further increased from 1.3 g/L to 2.6 g/L, the growth rate and biomass productivity continued to increase gradually. While, the lipid productivity remained constant between 1.3 g/L and 2.6 g/L nitrate concentration. Nonetheless, the lipid content was decreasing when nitrate concentration was increased from 0.65 g/L to 2.6 g/L.

Another similar study was performed by Li et al. (2008) on the lipid accumulation of *Neochloris oleoabundans* under different nitrate concentration (Figure 2-4). As shown in Figure 2-4, the highest lipid productivity of 0.133 mg/L·day was achieved at 0.425 g/L nitrate while the highest biomass productivity of 0.63 mg/L·day was achieved at 0.85 g/L nitrate respectively. It is also observed that with increasing nitrate concentration, the

lipid content, biomass productivity and lipid productivity were gradually decreased but the growth rate increased slightly.

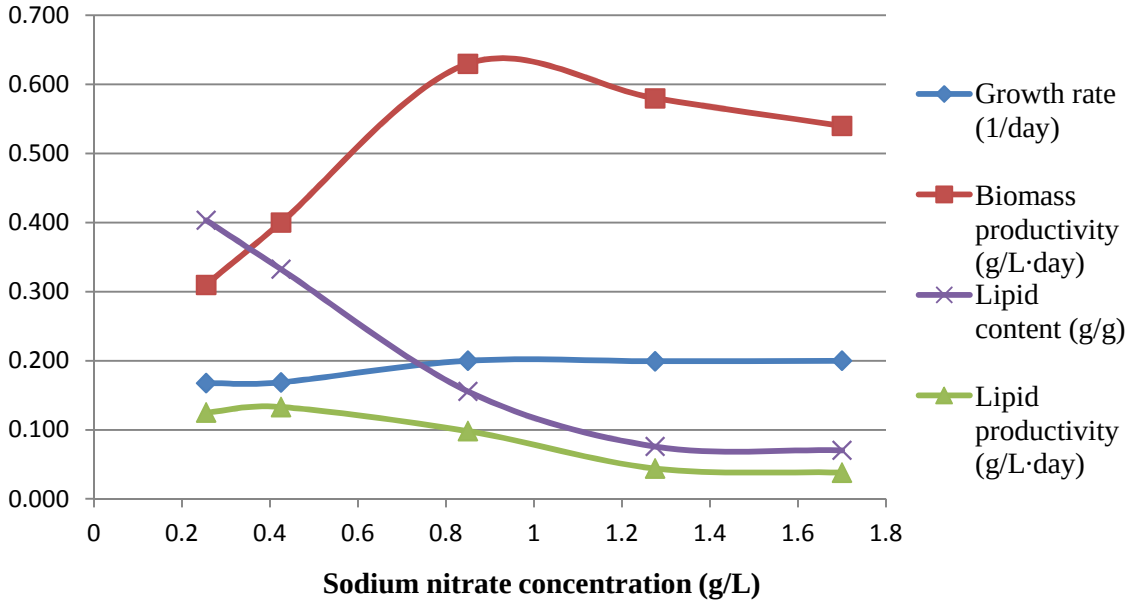

Figure 2-4: Growth rate, biomass productivity, lipid content and lipid productivity of *Neochloris oleobundas* under different sodium nitrate concentration (g/L) [52].

The different trend of growth response with increasing nitrate concentration showed that the highest lipid productivity and biomass productivity was not necessary contributed by maximum growth rate, biomass concentration or lipid content. The result clearly demonstrated that different nitrate concentration is required to achieve different objectives of the cultivation (optimum biomass productivity, lipid yield or lipid productivity). Therefore, the critical overall concentration of nitrogenous compound to be used for this study needs to be determined by the criteria of lipid productivity under observation of biomass productivity and lipid content.

Another interesting fact that has been widely published is that the quantity and quality of lipid from microalgae vary under the circumstance of nitrogen depletion [7,16,25,27,40,57,65,84,101]. Besides that, bio-diesel industry is favourable of gradual change of lipid composition, which involved conversion of protein into triacylglyceride

in microalgae under nitrogen deprivation [101]. The longer period of nitrogen depletion was associated with lower protein in lipid composition and higher lipid content.

Supportive research evidence was presented by Converti et al. (2009), 75% reduction in sodium nitrate concentration (1.5g/L → 0.375 g/L) in medium increased the lipid content (5.90% → 15.31%) and lipid productivity (8.16 mg/L·day → 20.30 mg/L·day) of *Chlorella vulgaris*. Similar outcome was also presented by Illman (2000) that *Chlorella vulgaris*, which was cultivated in low nitrogen medium, showed significant increase in lipid content (18% → 40%) and reduction in protein (29% → 7%). Widjaja et al. (2009) also showed the increment of biomass productivity (0.37 mg/L·day → 0.43 mg/L·day) and lipid productivity (9.75 mg/L·day → 12.77 mg/L·day) of *Chlorella vulgaris* between 15^{th} and 20^{th} cultivation day during nitrogen depletion. Besides that, Yeh et al. (2010) showed the highest lipid productivity of *Chlorella vulgaris* (39.55 mg/L·day) by the optimal bicarbonate concentration (1 g/L) with the supporting nutrient of 1.25 g/L nitrate concentration. At low nitrate concentration of 0.65 g/L, Chen et al. (2010) produced the highest growth rate and lipid productivity were 0.72 day^{-1} and 60.5 mg/L·day respectively. However, the maximum lipid content of 4.9% was obtained at 0 g/L nitrate concentration and the highest biomass productivity was 1.76 g/L·day at 2.6 g/L nitrate concentration. Generally, researchers showed that the growth inhibition and lipid accumulation were slowly induced as the microalgae advanced into the stage of nitrogen depletion.

In summary, Chen et al. (2010) showed the highest growth rate, biomass productivity and lipid productivity at 0.65 g/L potassium nitrate concentration from the studied range of 0 g/L to 2.6 g/L. Besides that, previous research done by Li et al. (2008) showed *Neochloris Oleobundas* microalgae cultivated in medium with sodium nitrate concentration, ranging between 0.255 g/L and 1.7 g/L. The optimum lipid productivity and biomass productivity was found at 0.425 g/L and 0.85 g/L sodium nitrate respectively. Another similar experiment was carried out by Converti et al. (2009) on *Chlorella vulgaris*. With the decrement of nitrate concentration from 1.5 g/L to 0.375 g/L, the biomass productivity was increased from 0.37 mg/L·day → 0.43 mg/L·day and

lipid productivity was increased from 9.75 mg/L·day to 12.77 mg/L·day. With the considerations to achieve optimum growth rate, biomass and lipid productivity, the proposed range of nitrate concentration for this study is between 0.5 g/L and 1.5 g/L in this study.

2.5.3 Effect of Light Irradiance in Cultivation

Light energy is one of the crucial factors in the photosynthesis process. Light energy can be measured with Photosynthetic Photonic Flux, PPF ($\mu mol \cdot m^{-2} \cdot s^{-1}$) or LUX [91]. The conversion factor of PPF to LUX varies with various types of light source, such as sunlight, cool white fluorescent lamp, high pressure sodium lamp and high pressure metal halide lamp.

In the previous research, the single effect of light irradiance on the microalgae was studied. The highest lipid productivity of 1.44 g/L·day and the highest lipid content of 1.4% was achieved at 12580 LUX for microalgae *Anabaena 7120* [3]. In the cultivation of *Chlorella vulgaris*, maximum biomass of 2.05 g/L was obtained at 4625 LUX and maximum lipid content of 33.38% was achieved at 7400LUX [46].

When *Nannochloropsis sp.* cultivation was cultivated under illumination of 51800 LUX, the highest lipid productivity of 0.41 g/L·day was achieved at 40 g/L sodium chloride and the maximum value of lipid content of 47% was achieved at 13g/L sodium chloride [68]. Pal et al. (2011) explained that *Nannochloropsis sp* was able to grow under stressed condition of high salinity in medium. Regardless of high salinity in medium, 51800 LUX which was considered as strong light irradiance, was able to be absorbed by *Nannochloropsis sp.* without any observation of photo-inhibition in Pal et al. (2011)'s experiment. On the other hand, in the work done by Carvalho et al. (2005), the highest lipid productivity of 0.267 g/L·day and lipid mass of 132.5 mg/L were obtained under 8880 LUX and 0.5% carbon dioxide from *Pavlona lutheri*.

Most of the previous studies reported that illumination of increased light irradiance will result in the increment of cell population, biomass [46,81] and lipid content [3,11,68].

However, excessive light irradiance or extremely low light irradiance will cause growth inhibition [12,46]. Sandnes et al. (2005) revealed that the specific growth of *Nannochloropsis oceanica* was increased when the light irradiance was increased from 2590 LUX to 5920 LUX but the study by Khoeyi et al. (2011) showed the decrement of growth rate of *Chlorella vulgaris* under further increment over 4625 LUX. Their study showed that the biomass increased as the light irradiance increased but decreased after 4625 LUX. However, the biomass productivity increased from 0.64 g/L·day to 2.32 g/L·day with increasing light irradiance from 2775 LUX to 7400 LUX. Archer (1997) showed that the lipid content of *Anabaena* was increased as the light irradiance was increased from 4884 LUX to 12580 LUX whereas Khoeyi et al. (2011) demonstrated that the lipid content of *Chlorella vulgaris* was decreased when the light irradiance was increased from 2775 LUX to 7400LUX. Compared to other microalgae, *Chlorella vulgaris* clearly showed different results when the light irradiance was increased. The decreasing trend of growth, biomass and lipid content in *Chlorella vulgaris* for increased light irradiance can be explained by the effect of photo-inhibition.

On the other hand, it is worth to note that during the cell growth, the light penetration is decreasing because of the increment of cell population [95]. Hence, at the high culture density, higher light irradiance is suggested to overcome the zone of insufficient light supply in the photo-bioreactor. However, high light irradiance will cause the growth photo-inhibition at low cell density culture. In order to achieve the goals of high cell growth with high lipid productivity, optimal light irradiance should be determined without provoking growth photo-inhibition at low and high culture density. Therefore, the range of light irradiance to be used in this study was proposed between 2000 LUX and 7000 LUX.

2.6 Modelling & Optimization

Since microalgae was acknowledged as the next potential substitute for bio-diesel and bio-ethanol industry, relatively high production costs of microalgae cultivation become major hindrance in microalgae production. In order to enhance productivity of microalgae cultivation and reduce production cost, it is important to fully understand the

productivity potential of lipid based microalgae by developing model to illustrate microalgae growth under different environmental condition.

A lot of efforts were put in developing different models to relate microalgae growth with various environmental condition based on different research objective. The works done by Di Toro et al. (1971) and James et al. (2010) showed a clear picture of the effect of investigated process parameters. The potential cause of eutrophication problem was investigated by developing dynamic model of phytoplankton population based on the concept of principles of conservation of masses [23]. The growth and death kinetic formulation for biological microorganisms have been empirically developed by analysis on existing experimental data. Another model was built to monitor microalgae growth in open-channel raceway under effect of atmospheric condition, water temperature, water column depth and flow rate [41]. From the obtained model, it is found out that high biomass concentration was obtained under microalgae cultivation of atmospheric condition of warm sunlight, water temperature of 20°C and water column depth of 60cm. However, change of flow rate did not show any remarkable improvement during observation. Although models developed by DiToro et al. (1971) and James et al. (2010) were very complex, these models were presented as a component in the solution for large scale experimental run.

One-factor-at-a-time (OFAT), which varies single factor while fixing others, has also been widely performed in laboratory scale cultivation of microalgae to illustrate the effect of selected factor in the cultivation. Bhola et al. (2011) investigated the effect of multiple factors on the biomass yield and thermal behaviour of *Chlorella vulgaris* by using OFAT. The optimised factors are 4% CO_2, 0.5 g/L NO_3^- and 0.04 g/L PO_4^{3-} with the responses of carbon fixation rate (6.17 mg/L·hr), lipid content (21%) and calorific value (17.44 kJ/g). Lv et al. (2000) used OFAT to optimise cultivation conditions in order to enhance lipid production of *Chlorella vulgaris*. With the optimised values of 1.0 mM KNO_3, 1.0% CO_2 and 60 $\mu mol \cdot m^{-2} \cdot s^{-1}$, the highest of lipid productivity of 40 mg/L·day was obtained. Unfortunately, both experimental results failed to demonstrate the interaction between factors using OFAT.

White and black box approaches are able to analyse multiple factors simultaneously and shows more accurate result compared to OFAT. Also, white or black box approach can develop model to identify interaction between factors affecting microalgae growth. Although white box approach can show more promising result compared to black box, white box approach is not applicable in this study due to limited knowledge on cultivation obtained from literature review on *Chlorella vulgaris* from CSIRO. Besides that, *Chlorella vulgaris* growth was investigated under the effect of medium composition and light irradiance, without taking photosynthesis mechanism and photo-bioreactor design into consideration. Also, all environmental conditions were controlled within desired range in this study when the microalgae cultivation was carried out in laboratory. Hence, black box approach is more appropriate approach in model developing.

Factorial experimental design, which is one kind of black box approach, is able to analyse multiple factors simultaneously and shows more accurate result compared to OFAT. The comparison between OFAT and factorial experiment is shown below in Table 2-1.

Table 2-1: Comparison between the one-factor-at-a-time and factorial experiment [18].

OFAT	**Factorial Experiment**
More runs are required to achieve the precision of single effect.	Few steps are required to attain the precision of multiple effects simultaneously.
Interactions cannot be studied between parameters	Interactions between parameters are clearly displayed in term of significance.
Optimal configuration might be missed.	Optimal configuration can be successfully developed.

From the comparisons, it is concluded that factorial experiment is a more appropriate statistical approach to build a model that can achieve the goals of investigating the interaction between parameters and formulating the optimal configuration. However, factorial experiment can be applied to the process in which the continuous factors must

be linear effects. If quadratic effect is shown by a factor, response surface methodology (RSM), which is second degree polynomial experiment, such as central composite design, Box-Behnken design and so on, should be applied.

For the models generated from RSM, the interaction between factors can be identified by MANOVA. The benefit of MANOVA with statistical significance threshold is to ensure developed model are valid with the criteria of model validity, each single effect on response, each interaction between factors on response and lack of fit. This is the advantage of generating model using RSM compared to OFAT because combination effect of the investigated factors can be fully understood from the generated model and optimal configuration can be easily obtained from the model with less experimental runs compared to OFAT method.

With the understanding of relationship between variables and response from developed model, the cultivation outcome can be enhanced by applying mathematical optimization. Mathematical optimization is an important tool to generate optimal configuration for maximising the microalgae production in this study. Simplex algorithm is one of the popular algorithm in searching for optimal configuration [19,20,64,92]. The simplex algorithm is a hill climbing algorithm to search for a vector of parameters which generates maximum or minimum response in the objective function within the range of parameters.

Li et al. (2011) applied the Box-Behnken design into optimization of the biomass production of *Chlorella minutissima* UTEX2341. When the cultivation parameters were controlled at 26.37 g/L·day carbon (A), 2.61 g/L·day nitrogen (B) and 0.03 g/L·day phosphorus (C), highest biomass productivity of 1.78 g/L·day was achieved. The mathematical regression model for biomass productivity fitted in terms of actual factors is as shown below:

$$\textit{Biomass productivity} = 1.4681 + 0.4740\,A + 0.0330\,B - 0.0537\,C - 0.0299\,AB - 0.0028\,AC - 0.2334\,BC - 0.2046\,A^2 - 0.2072\,B^2 - 0.0374\,C^2 \quad (2\text{-}6)$$

On the other hand, preliminary work performed by Rajasri et al. (2013) identified that light intensity and sodium nitrate concentration had influence on biomass production of *Chlorella pyrenoidosa* by employing Plackett-Burman design. These parameters were further optimized by employing central composite design. Maximum biomass yield of 2.956 g/L was obtained at 1.78 g/L nitrate concentration (A) and light intensity (B) of 7062 LUX. Besides that, the effect of light irradiance and sodium nitrate concentration on biomass production of *Chlorella pyrenoidosa* is illustrated by developing a quadratic model, as shown below.

$$\textit{Biomass concentration} = 2.49 + 0.18\,A + 0.99\,B - 0.0011\,AB + 0.081\,A^2 - 0.76\,B^2 \qquad (2\text{-}7)$$

Another optimization on lipid production of *Chlorella sp.* was performed by applying response surface methodology in order to maximise lipid productivity [104]. A second order polynomial equation was generated from central composite design to illustrate the effect of temperature, sodium nitrate concentration and glucose concentration on lipid production. MANOVA showed that interaction between glucose and sodium nitrate concentrations, and interaction between sodium nitrate concentration and temperature, contribute effect on lipid production of *Chlorella sp*. The highest lipid productivity of 247.16 mg/L·day was noticed at the optimal parameters which were 26.2 g/L glucose concentration (A), 2.06 g/L sodium nitrate concentration (B) and temperature (C) of 28.18 °C. The second order polynomial equation is as displayed below.

$$\textit{Lipid productivity} = 220.72 + 55.57\text{A} + 34.08\,B - 11.70\,\text{C} + 29.07\,AB + 2.42\,\text{AC} + 14.20\,\text{BC} - 56.90\,A^2 - 11.67\,B^2 - 53.03\,C^2 \qquad (2\text{-}6)$$

In biological research, many researchers began to apply RSM into their work and RSM is getting popularity in optimization of experiment configuration. Although there is limited knowledge on the research problem, applying RSM with a few experimental runs can develop model to illustrate the research problem under effect of selected parameters. Among RSM, central composite design is one of the useful and efficient experimental

tools to provide information on effects of experimental variables and overall experimental error with minimum number of experimental runs.

Central composite design consists of full factorial design with centre point (red dots) which is augmented with a group of axial points (green dots), as shown in Figure 2-5 [8]. With the addition of green dots, central composite design is able to estimate regression of all investigated parameters required to develop a second order mathematical model with respected to a designated response.

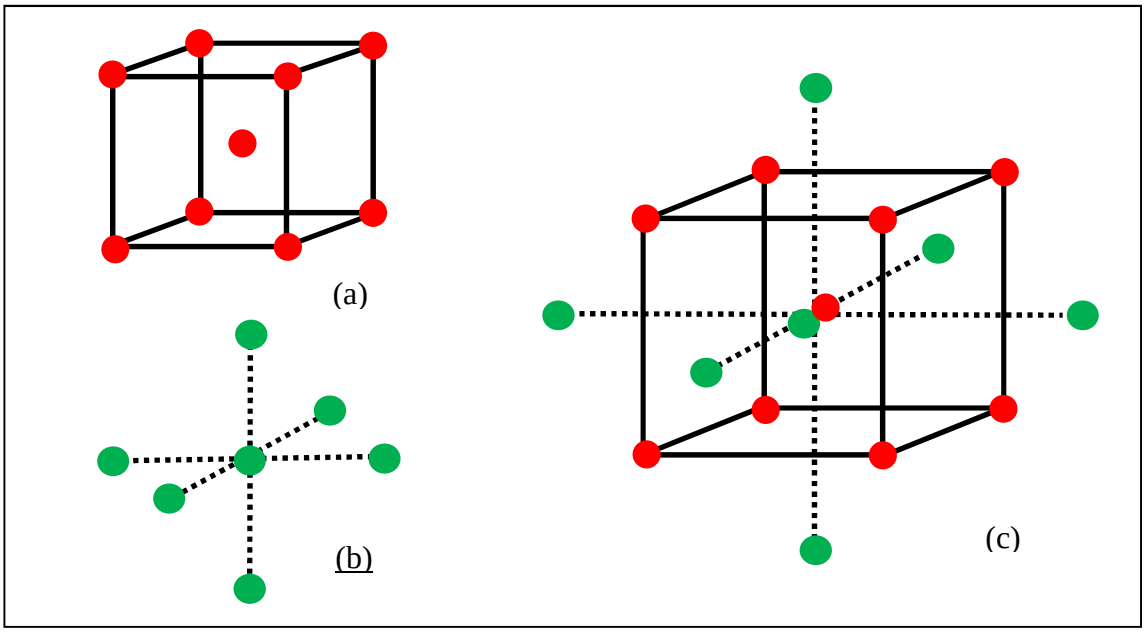

Figure 2-5: Experimental design with 3 factors. (a) Factorial design with centre point. (b) A group of axial points. (c) Central composite design circumscribed.

On the other hand, Box Behnken design is also quadratic experimental design which does not consist of factorial design. As shown in Figure 2-6, it is shown that only middle points between the edges are studied in Box Behnken design. This experimental design has limited capability for orthogonal blocking compared to central composite design.

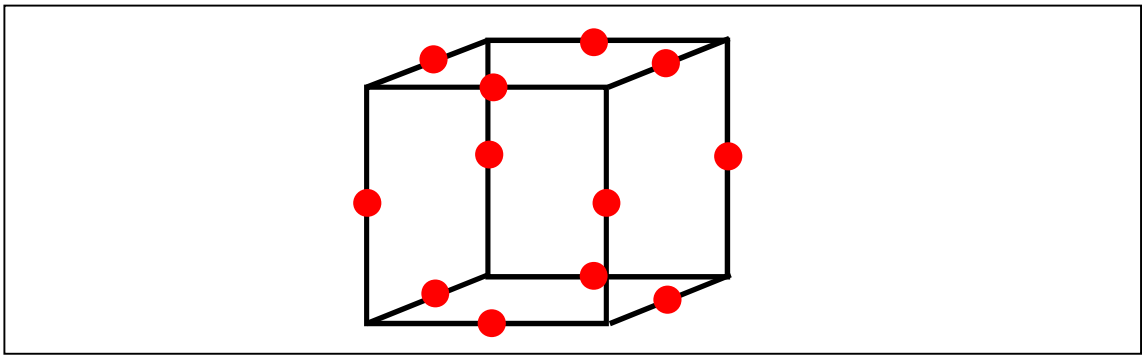

Figure 2-6: Box Behnken design for 3 factors.

Therefore, in order to generate second order polynomial model, central composite design instead of Box Behnken design is proposed to be applied in this study to investigate proposed environmental conditions on microalgae growth. On the other hand, simplex optimization is applied on the obtained mathematical model to produce optimal configuration for maximum cultivation outcome.

Two research works was found to be similar with this study [13,105]. Chen et al. (2010) investigated lipid productivity of *Chlorella vulgaris* under effects of bicarbonate and nitrate concentrations. On the other hand, biomass productivity of *Chlorella vulgaris* was studied by Yeh et al. (2010) under variation of bicarbonate concentration, nitrate concentration and light irradiance. The maximum productivity of *Chlorella vulgaris* was obtained by applying OFAT method. Both studies showed that none of the model was generated and interaction between investigated environmental conditions was unable to be identified. In order to develop models to illustrate microalgae growth and identify interaction between environmental conditions, central composite design was applied to investigate growth rate, biomass productivity and lipid productivity of *Chlorella vulgaris* under effects of bicarbonate concentration, nitrate concentration and light irradiance. With the three models obtained, an optimized process configuration can be generated to maximize productivity of *Chlorella vulgaris*.

2.7 Conclusion & Specific Objectives for the Present Study

From the review of the literature, it is found out that most literatures discussed the single response of cultivation with varying cultivation condition [7,12,16,101]. This however, could have neglected some important facts of other interactive responses. To date, very few studies were performed to study the combined effects of different environmental conditions on the growth, biomass productivity, as well as lipid productivity of microalgae.

Furthermore, in the past, many researchers used OFAT method to study the single effect of the parameter on the cultivation. Unfortunately, the application of OFAT method fails to analyse the important relationship between chosen factors because the combination of factors might be able to improve the outcome of the study.

In this study, *Chlorella vulgaris* growth was studied under effect of medium composition and light irradiance without taking photo-bioreactor design and photosynthesis mechanism into consideration. Due to the limitation of this study, black box approach was more applicable approach compared to white box approach. Therefore, central composite design of black box approach, with statistical analysis of multivariate analysis of variance (MANOVA), was applied in this study in order to clearly illustrate the interactions between factors.

In order to regulate the cultivation of *Chlorella vulgaris* effectively, optimization is necessary to shorten the cultivation period, maximise the growth and promote the lipid production of microalgae. This can maximise the profit in order to reduce the high expense of the cultivation. Until now, very few of previous researchers have performed optimization on model developed. Hence, it is worth to perform optimization on the cultivation of *Chlorella vulgaris* in this study.

In this research, three responses of the experiments (growth, biomass productivity and lipid productivity) were investigated by manipulating three factors: bicarbonate concentration, nitrate concentration and light irradiance. Carbon dioxide is very important cultivation condition in the growth phase. However due to the limited solubility of carbon dioxide in water, supplying carbon dioxide gas to the culture is not a sustainable way because only partial of carbon dioxide gas will dissolve in the water and the remaining carbon dioxide gas will escape to atmosphere. As mentioned earlier, the bicarbonate ion is able to maximise concentration of aqueous carbon dioxide due to the chemical equilibrium. Hence, bicarbonate concentration is the first chosen factor that will be studied in this research. Nitrate concentration is selected as second factor because it was reported that nitrate is able to boost the growth of microalgae [56,59,94]. Light irradiance was chosen as the third factor to be studied because light is the compulsory element in the photosynthesis of the microalgae.

The common point between these selected factors is that they are all essential elements for the photosynthesis of microalgae. On the other hand, growth, biomass productivity and lipid productivity were chosen as responses and three models were built in order to demonstrate interaction between factors with respect to different response. Finally, optimization will be carried out in order to generate the optimized experiment configuration to improve the cultivation of *Chlorella vulgaris*.

Therefore, the research objectives are derived from the major scopes:

- To identify individual and combinational effects of bicarbonate, nitrate concentration and light irradiance on growth rate, biomass productivity and lipid productivity of *Chlorella vulgaris*.

- To develop models, which predict the growth rate, biomass productivity and lipid productivity of *Chlorella vulgaris*

- To determine optimal environmental conditions for lipid productivity with the aid of simplex optimization.

Chapter 3 Methodology & Analysis Technique

3.1 Introduction

This chapter outlines the research methodology, experimental approach and analysis technique that were used to achieve the objectives of this research study. The overview idea of methodology was briefly summarized in the Figure 3-1 and the details of methodology were clearly explained in this chapter.

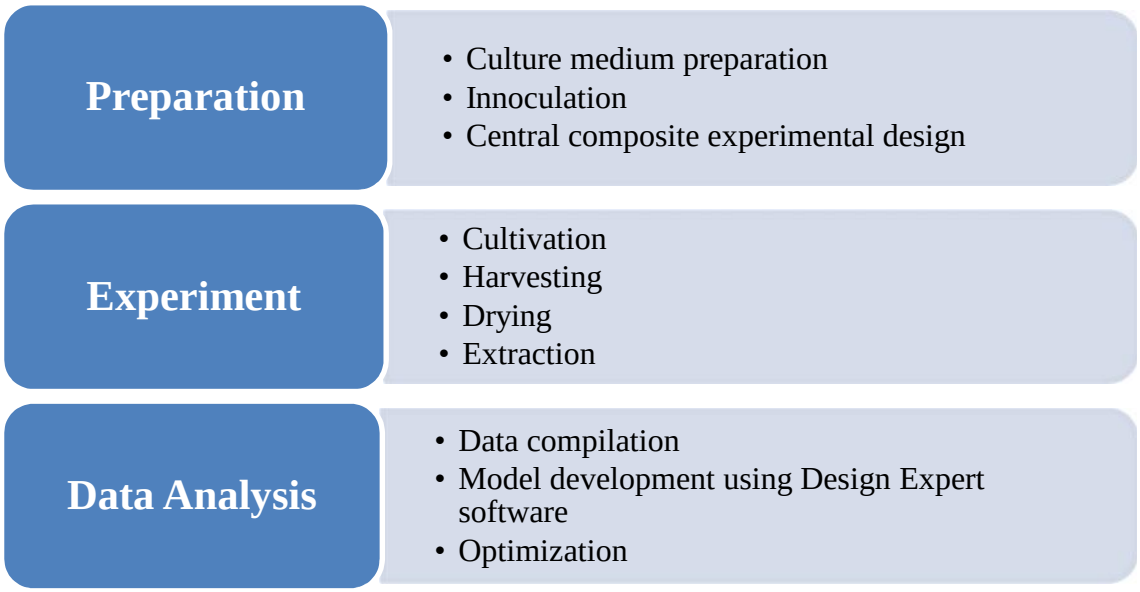

Figure 3-1: Methodology flow chart

In this study, central composite design was applied to perform experimental design, which decides the number of sets of experimental runs and combination of environmental conditions for each run. Culture medium was prepared for inoculation and cultivation of microalgae. Inoculation was carried out to maintain stock culture healthy and sub-culture *Chlorella vulgaris* for the experimental runs. This is followed by microalgae cultivation, cell counting, harvesting, drying and lipid extraction. The collected data were further analyzed using multivariate analysis of variance (MANOVA) to develop mathematical model. With obtained model, process configuration for maximum lipid productivity can be obtained by simplex optimization.

3.2 Microalgae Culture, Medium and Chemicals

Chlorella vulgaris strain was obtained from Commonwealth Scientific and Industrial Research Organisation (CSIRO), Australia. The strain was cultivated in the modified Bold's Basal Medium (BBM) [1] which is recommended for *Chlorella* strain. The medium composition and final concentration of each chemical in medium are shown in Table 3-1.

Table 3-1: Recipe for 1 litre of modified Bold's Basal Medium [1]

Component	Stock solution (g/L)	Quantity Used (ml)	Concentration in Final Medium (mol/dm^{-3})
Macronutrients			
$NaNO_3$	25.0	10	2.94×10^{-3}
$CaCl_2 \cdot 2H_2O$	2.5	10	1.70×10^{-4}
$MgSO_4 \cdot 7H_2O$	7.5	10	3.04×10^{-4}
K_2HPO_4	7.5	10	4.31×10^{-4}
KH_2PO_4	17.5	10	1.29×10^{-3}
NaCl	2.5	10	4.28×10^{-4}
Micronutrients			
EDTA	50.0	1	1.71×10^{-4}
$FeSO_4 \cdot 7H_2O$	5.0	1	1.79×10^{-5}
Reverse Osmosis Water		938	

Note: 1. EDTA stands for ethylenediaminetetraacetic acid

2. All chemical belong to laboratory grade chemical (Bendosen, Malaysia).

Initially, with the use of reverse osmosis water, 1L stock solution for each macronutrient and micronutrient was prepared and stored in glass bottle stored inside the laboratory chiller. In order to prepare 1 litre of BBM, 10ml and 1ml were taken from each macronutrient and micronutrient stock solution respectively and reverse osmosis water was added to obtain medium with total volume of 1L.

3.3 Sub-culturing

In microalgae cultivation, sub-culturing is a very important approach in initiating large scale cultivation and maintaining healthy stock culture. Stock culture, which must be maintained weekly, is kept in 500ml conical flask covered by cotton bung.

Initially, 20ml of stock culture was inoculated in a 250ml aerated conical flask containing 100ml of Bold Basal Medium (For recipe, please refer to Section 3.2) under 4500 LUX cool daylight (Colour temperature: 6200K) fluorescent lamp [30]. At day zero of *Chlorella vulgaris* cultivation or sub-culturing, approximately 10^5 cell/ml was counted for the cell count. After two weeks, the 120ml inoculants were transferred into 1500ml aerated conical flask and inoculated with 600ml of Bold Basal Medium. Similarly after two weeks, a total of 720ml of inoculants were successfully sub-cultured. Out of 720ml inoculants, 220ml was used to prepare new stock culture, while remaining 500ml was used for 3 L culture medium experiment respectively.

When the microalgae sample was ready, sub-cultured sample was harvested by centrifugation at 3500 rcf (relative centrifugal force) for 10min to remove the solution. After that, the collected residue of microalgae was washed with reverse osmosis water few times in order to remove remaining medium solution. The residue of microalgae was then diluted in reverse osmosis water to be used as the feed of the further experiments or stock culture.

In general, the volume of sub-culturing was increased by approximately 5 times for each sub-culturing process. The sub-culturing process was repeated every 2 weeks in order to prepare sample for large scale cultivation or maintain stock culture healthy.

3.4 Experimental System with Photo-bioreactor

Chlorella vulgaris was cultivated in a flat panel photo-bioreactor (40cm length, 10cm width and 30cm height) with 3L of culture medium. The experimental set up is illustrated in Figure 3-2. Continuous lightning was supplied to each side of photo-bioreactor using cool daylight fluorescent lamp (6200K). The culture medium was aerated continuously using air sparger at a rate of 3.5L/min air. With continuous aeration using air sparger, microalgae was allowed to move from low light exposure region to brighter region to absorb light irradiance. Besides that, aeration could prevent microalgae agglomeration in culture medium inside photo-bioreactor.

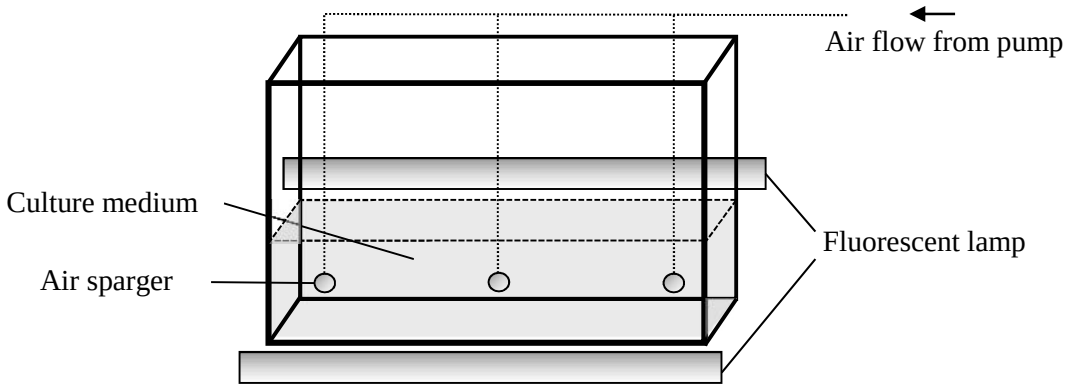

Figure 3-2: Schematic representation of photo-bioreactor

3.5 Experimental Design of Batch Cultivation

The key environmental conditions were sodium nitrate concentration (g/L), potassium bicarbonate concentration (g/L) and light irradiance (LUX) in this study. On the other hand, the selected responses of the design were growth rate (day^{-1}), biomass productivity (mg/L·day) and lipid productivity (mg/L·day). In this study, central composite design was implemented to design the experimental design. The levels of the environmental conditions for this study were shown in Table 3-2. Average value was obtained by repeating each experimental run twice. The results were analysed using MANOVA.

Table 3-2: List of experiment configurations developed from central composite design

Environmental condition 1 A:Bicarbonate (g/L)	Environmental condition 2 B:Nitrate (g/L)	Environmental condition 3 C:Light irradiance (LUX)
2.5	0.5	7000
7.5	1.5	2000
7.5	0.5	7000
2.5	0.5	2000
5	1	4500
5	1	4500
7.5	1.5	7000
2.5	1.5	2000
2.5	1.5	7000
7.5	0.5	2000
5	0.16	4500
5	1	4500
5	1	296
0.8	1	4500
5	1	8704
9.2	1	4500
5	1.84	4500
5	1	4500

3.6 Preparation for Varied Environmental Condition

As discussed in section 2.5, experimental parameters involved in *Chlorella vulgaris* cultivation can be divided into three categories which are fixed variable, adjustable variable, and non-fixed or non-adjustable variable. A list of experimental parameters involved in *Chlorella vulgaris* cultivation was displayed in Table 3-3. Light irradiance, sodium nitrate concentration and potassium bicarbonate concentration were the environmental conditions to be varied during cultivation of microalgae.

Table 3-3: Experimental parameters in this study.

Non-Fixed or Non-Adjustable Variable	**Fixed Variable**	**Adjustable Variable**
pH	Medium temperature 30°C	Bicarbonate concentration
	24 hours illumination period	Nitrate concentration
	Initial cell count 10^5 cell/ml	Light irradiance

3.6.1 Potassium Bicarbonate Concentration

Bicarbonate concentration plays important role in supplying carbon source to produce high organic molecule in carbon fixation phase. In order to investigate the effect of higher bicarbonate concentration on *Chlorella vulgaris* cultivation, the amount of potassium bicarbonate powder added into culture medium was varied. As discussed in section 2.5.1, the studied range for potassium bicarbonate concentration was proposed between 2.5g/l and 7.5g/L.

Initially, potassium bicarbonate powder was weighed on a weighing paper by using analytical balance (Sartorius SECURA213-1ORU). After that, weighed potassium bicarbonate powder was poured into culture medium contained in the photo-bioreactor. The weighing paper was weighed again in order to calculate mass of potassium bicarbonate powder poured into culture medium. The culture medium was stirred by using glass rod to sure potassium bicarbonate powder is completely dissolved in culture medium.

3.6.2 Sodium Nitrate Concentration

Nitrate concentration is another studied environmental condition which can enhance the growth of *Chlorella vulgaris*. The effect of higher nitrate concentration was investigated by varying amount of sodium nitrate added to culture medium. As discussed in section 2.4.2, the lower and upper limit boundary for sodium nitrate concentration were proposed as 0.5g/L and 1.5g/L respectively.

Initially, analytical balance (Sartorius SECURA213-1ORU) was used to weigh sodium nitrate powder on a weighing paper. The weighed sodium nitrate powder was then poured into culture medium contained in the photo-bioreactor. After that, the weighing paper was weighed again to obtain total mass of sodium nitrate powder poured into culture medium. Glass rod was used to stir culture medium with sodium nitrate powder to ensure it is dissolved completely in culture medium.

3.6.3 Light Irradiance

In this study, light irradiance is one of the environmental conditions to be varied during cultivation. As discussed in section 2.5.3, the proposed lower and upper limit for light irradiance was between 2000LUX and 7000LUX.

According to inverse square law for light, it states that light irradiance is inversely proportionally to the square of the distance from the light source. In order to acquire the desired value of light irradiance, the distance between fluorescent lamp and external surface of photo-bioreactor or sub-culturing flask was adjusted. In this study, the reading of light irradiance was measured by light meter (TENMA 72-6693).

When both sides of photo-bioreactor were illuminated by two fluorescent lamps (as shown in Figure 3-2), both fluorescent lamps were placed facing toward the photo-bioreactor. The sensor cover of light meter was removed and the light sensor was placed next to the side of photo-bioreactor. In order to obtain desired value of light irradiance, the distance between fluorescent lamp and photo-bioreactor was adjusted by moving the position of fluorescent lamp. Desired value of the light irradiance was measured along the illuminated surface of photo-bioreactor to ensure consistency of light exposure along the illuminated surface of photo-bioreactor.

3.7 Cell Counting and Measurement of Growth Rate

To obtain growth rate of microalgae, cell counting needs to be performed. When the cultivation was started, the cell count was carried out daily until the stationary phase was reached, which was identified when the numbers of cells count remained constant for three consecutive days. In this study, cell counting was carried out three times for each sample every 24 hours in order to obtain the average cell count.

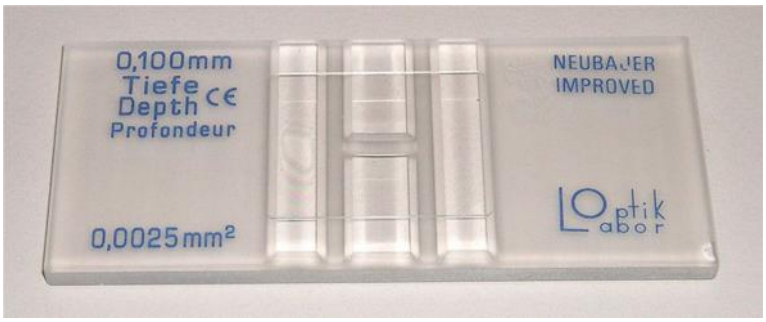

Figure 3-3: Improved Neubauer haemocytometer [2].

Improved Neubauer haemocytometer (Figure 3-3) was used to perform a direct microscopic count on the sample of cultivation. Cover slip was gently placed on the surface of haemocytometer and cover slip was centred over the counting chamber [74]. Cultivation medium in the photo-bioreactor was stirred before the sample was taken to ensure homogeneity of the cells in the medium. A small amount of sample (approximately 3ml) was taken from 3L cultivation medium by using fine tipped Pasteur pipette. One drop of sample (approximately 0.05ml) was drawn out from the pipette and placed at the edge of cover slip. The sample was distributed into the counting grid area of haemocytometer by surface tension of solution. The counting grid of haemocytometer (Figure 3-4) can be viewed clearly under microscope.

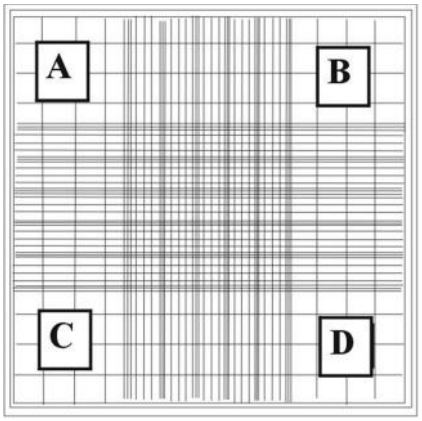

Figure 3-4: Counting grid of improved Neubauer haemocytometer [74].

When the concentrated sample was obtained, it is necessary for the sample to be diluted with pure water in order to reduce the risk of inaccurate counting. The amount of pure water that was added for dilution needs to be taken into consideration because it will

affect the dilution factor. When the cell population was rapidly increasing, more pure water was required to dilute the concentrated sample, which results in higher dilution factor. During cell counting, the cells in area A, B, C and D are taken into consideration [1,74] and the number of cells per ml is calculated by using Equation 3-1.

$$\text{Cells per ml} = \frac{\text{number of cells}}{\text{4 squares}} \text{ x dilution factor x } 10^4 \quad (3\text{-}1)$$

With the assumption of exponential growth during the growth phase of cultivation, the specific growth rate, μ (day^{-1}) was calculated using (3-2).

$$\mu = \frac{\ln N_t/N_0}{\Delta t} = \frac{\ln N_t - \ln N_0}{t_t - t_0} \quad (3\text{-}2)$$

where N_0 and N_t are the cell count at the beginning and end of a time interval respectively. Δt is the length of time interval $t_t - t_0$.

3.8 Harvesting and Measurement of Biomass

As the cell count of microalgae cultivation was continuously maintained constant for 3 consecutive days, the cultivation entered the stationary phase and microalgae were ready to be harvested. 3L of cultivation medium was harvested by centrifugation (Heraeus, Labofuge 400 millilitre - 1 litre) at 3500 rcf (relative centrifugal force) for 10min. The suspended sample was washed with reverse osmosis water and the mixture of water and sample was centrifuged at 3500 rcf. Washing and centrifugation were repeated twice in order to remove remaining salt in the residue.

Work done by Widjaja et al. (2009) showed that different drying temperature could affect the lipid content of *Chlorella vulgaris*. Drying temperature of 80˚C and 100˚C were found to result in decrease in lipid content of *Chlorella vulgaris*. On the other hand, lipid content was slightly decreased under drying temperature of 60˚C. However, drying under temperature of 60˚C was not efficient drying process because longer time was needed to remove water completely from wet biomass. Since same lipid content was

showed under drying temperature of 80˚C and 100˚C, drying temperature of 80˚C is preferable as it is more energy saving and reduces utility cost comparably.

Dry filter paper was initially weighed on analytical balance (Sartorius SECURA213-1ORU). The collected residue from centrifugation was placed on the filter paper. The wet biomass on filter paper was dried in the oven under temperature of 80˚C for 24 hours. The dried sample with filter paper was weighed by using analytical balance (Sartorius SECURA213-1ORU) and dried biomass mass (mg) was obtained.

3.9 Lipid Extraction and Measurement of Lipid Mass

Solvent extraction was used to extract lipid from the dried sample into organic solvent using Soxhlet method. Soxhlet extractor (Figure 3-5) is an unique equipment that allows solvent staying inside extraction chamber for a short period.

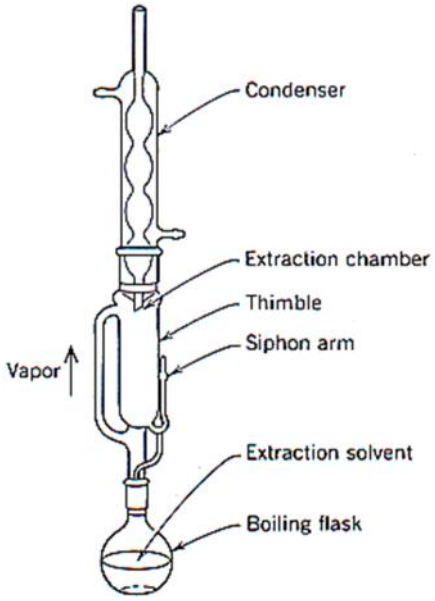

Figure 3-5: Soxhlet extractor apparatus [88].

The empty 500ml boiling flask with boiling chip was weighed on analytical balance (Sartorius SECURA213-1ORU). After that, the boiling flask was filled with 350ml hexane. The apparatus for solvent extraction was set up by connecting boiling flask, Soxhlet extractor and condenser. The boiling flask with hexane and boiling chip was

heated up to 200 ˚C for 8 hours on digital hot plate (IKA C-MAG-HS-7). The vaporized hexane entered the condenser tower and the condensed hexane flowed back into extraction chamber. After that, when the solvent reached certain level, it was siphoned back into the boiling flask and was heated again. This cycle was repeated for few hours in order to extract the lipid from microalgae and collect the extracted lipid in boiling flask.

After Soxhlet extraction using hexane as solvent was performed under temperature of 200˚C for 8 hours, a solution mixture of lipid and hexane was obtained. In order to separate lipid from hexane, boiling flask with mixture of lipid and hexane was heated on digital hot plate (IKA C-MAG-HS-7) under temperature of 200˚C for 10 minutes. Hexane vapour produced from boiling flask was condensed at the wall of glass tube of Liebig condenser (as shown in Figure 3-6) which is surrounded by a glass envelope through which cooling water flows. After hexane was removed, the lipid was left in the boiling flask as extract and the extract was analyzed by using gas chromatography (Agilent GC 6890) to identify if there is remaining hexane. After hexane was completely removed, the boiling flask with extract and boiling chip was weighed on analytical balance (Sartorius SECURA213-1ORU). The lipid mass (mg) extracted from dried biomass was determined from the comparison of weighed boiling flask before hexane extraction and after hexane recovery.

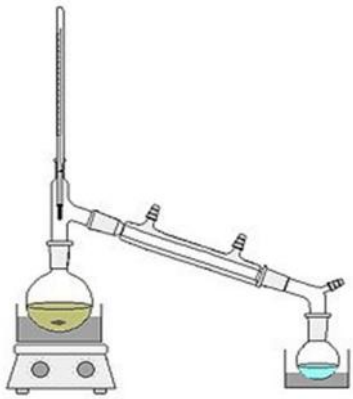

Figure 3-6: Recovery of organic solvent by using Liebig condenser [98].

3.10 Lipid Productivity

Using the data obtained from growth rate (day^{-1}), moisture free biomass mass (mg) and lipid mass (mg), the biomass productivity (mg/L· day) and lipid content (mg/mg) were generated in order to determine the lipid productivity (mg/L· day). All the equations (Equation 3-3 to Equation 3-7), which are required for the calculation of lipid productivity were obtained from the literature article written by Griffiths and Harrison (2009).

Biomass concentration (3-3) is defined as the dry biomass mass per volume of culture medium.

$$\text{Biomass concentration} = \frac{\text{Dry biomass mass}}{\text{Volume of culture medium}} \quad (3\text{-}3)$$

Biomass productivity (3-4) is the product of biomass concentration and specific growth rate.

$$\text{Biomass productivity} = \text{Biomass concentration} \times \text{Specific growth rate} \quad (3\text{-}4)$$

Lipid concentration (3-5) is the amount of lipid mass per unit volume of culture medium.

$$\text{Lipid concentration} = \frac{\text{Lipid mass}}{\text{Volume of culture medium}} \quad (3\text{-}5)$$

Lipid content (3-6) is the ratio of lipid concentration to biomass concentration.

$$\text{Lipid content} = \frac{\text{Lipid concentration}}{\text{Biomass concentration}} \quad (3\text{-}6)$$

Lipid productivity (3-7) is calculated using biomass productivity and lipid content.

$$\text{Lipid productivity} = \text{Biomass productivity} \times \text{Lipid content} \quad (3\text{-}7)$$

3.11 Statistical Analysis (MANOVA) – Overview

Figure 3-7 displayed the flow of verification and validation of generated model. Model verification and validation are very important steps in the model building sequence. As shown in Figure 3-7, all the obtained experimental data were analyzed using multivariate analysis of variance (MANOVA) approach to identify the interactions between factors with the effect. From the MANOVA result, a mathematical model can be developed and the interaction of environmental conditions can be identified.

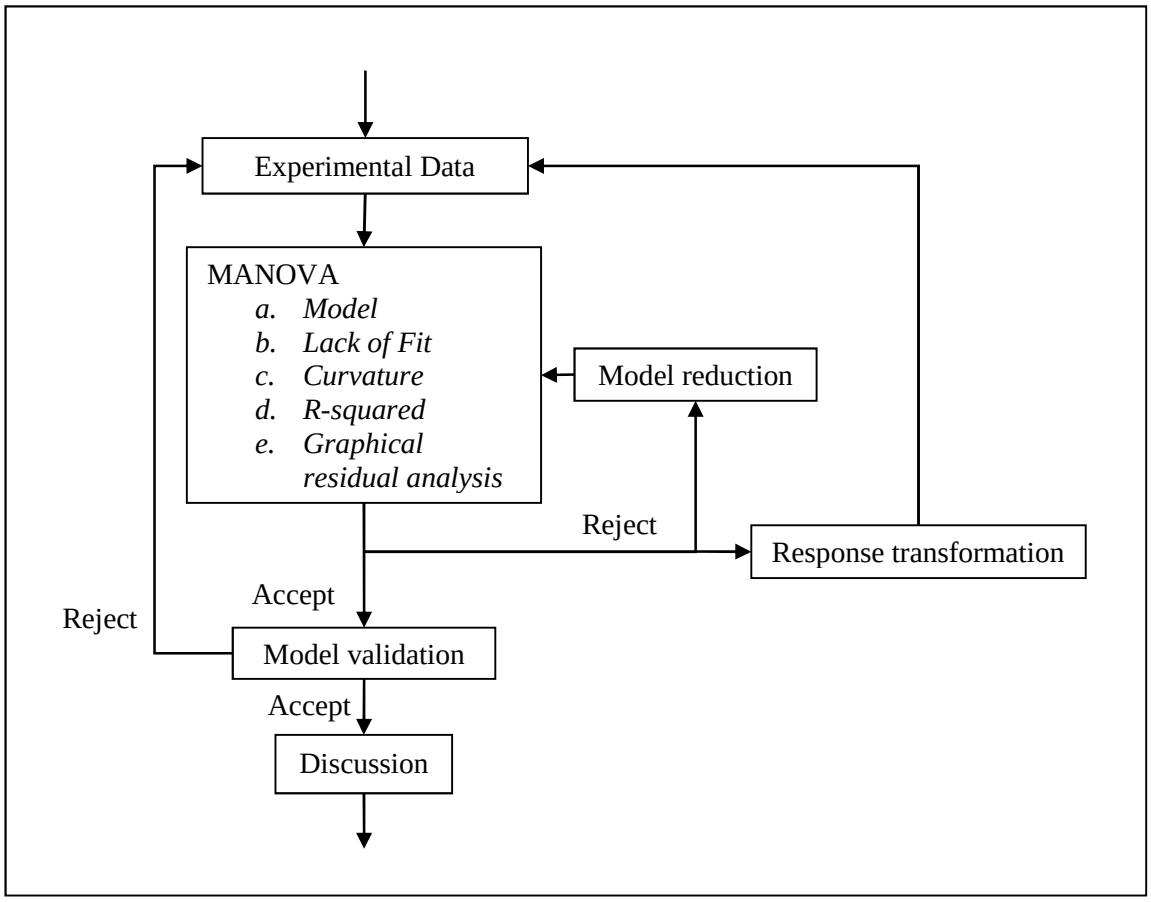

Figure 3-7: Flow of verification and validation of generated model.

Model verification is carried out to ensure the developed model fits the data well by achieving certain specifications. For statistical purposes, it is assumed that the residuals are normally distributed and independent with constant variance in MANOVA. The generated model can be verified by the sources of MANOVA, which are Model, Lack of

Fit and Curvature. In this study, significance threshold of MANOVA is set in order to maintain model precision. The probability value of the source (Model, Lack of Fit and Curvature) must be lower than significance threshold in order to produce significant source. Model source indicates that the validity of developed model, which is made up of different model terms in the order of hierarchy. On the other hand, Curvature source implies whether the order of the model is appropriate and Lack of Fit shows the measures of how well the model fits the data. Besides that, R-squared also provides the measure of how accurate the predicted value matches with the original data points. If any of MANOVA source is identified as insignificant, model reduction should be considered in order to improve MANOVA result.

Graphical residual analysis is also efficient tool for the model verification and the typical of graphical residual analysis are normal plot of residuals and residuals versus predicted level. The residual is defined as the difference between predicted value and experimental data. Graphical residual analysis mainly verifies the assumptions whether the residuals are approximately normal distributed and independent with constant variance. In normal plot of residuals, small deviation between residuals and straight line of normality implies that the residuals support the assumption of normality. On the other hand, the residuals are independent with constant variance when the residuals do not form any organized or systematic pattern and scatter thoroughly in residuals versus predicted plot. If the model fails to meet the assumptions, response transformation is suggested to treat the experimental data.

Although a model is built on the basis of high value of R-squared and non-significant Lack of Fit, this cannot ensure that the model fits the data well. Therefore, in this study, after the precedence experimental runs, same amount of experimental runs with different experimental configuration were carried out to check if experimental data are close to the predicted value from developed mathematical model.

In this study, central composite design will be applied to study *Chlorella vulgaris* growth. The quadratic model (Equation 3-9) was suggested to investigate the interactions of the environmental conditions with respect of selected response.

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{12} AB + \beta_{13} AC + \beta_{23} BC + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{123} ABC \quad (3\text{-}9)$$

where, *A*, *B* and *C* are the factors and *Y* is the response.

3.12 Response Surface Methodology

Response surface methodology (RSM) can be defined as a statistical tool that uses the result of the designed experiments to explore the relationships between multiple factors and responses. The main idea of the RSM is typically to produce optimum operating condition. Second-degree polynomial model was suggested for the purpose of optimization [10].

Main approach of RSM is to develop second order polynomial model by applying central composite design. This approach is sufficient to identify which factors contribute impact on the responses. Besides that, second-order polynomial model was developed from central composite design for optimization.

3.13 Optimization

Optimization generally consists of maximizing or minimizing an objective function by selecting the input values from within desired range and generating the optimum value of the objective function. In this study, simplex optimization is applied to maximise the lipid productivity of microalgae by applying central composite design. Simplex optimization is a hill-climbing algorithm in the search of the vector of parameters leading to the global extreme of n-dimensional function, searching though the designated range of environmental conditions.

A mathematical model function y = f (x_1, x_2, …, x_n) of N variables x= {x_1,x_2, …, x_n} is developed. The goal is to find local maximum y_{max} of this function corresponding variables x. After having generated the first simplex, the best point B (best response), SW (second-worst response) and W (worst response) are determined. Then, the position of centroid, CEN, which between points B (best response) and SW (second-worst response), is calculated.

In simplex method, there are four methods which are used repeatedly until the best point is obtained. The first step to generate a new simplex is the reflection of the worst point at the centroid. Then, three other methods are also applied to construct a new simplex:

- The expansion to accelerate the reduction of the simplex
- The contraction to keep the simplex small, and
- The compression around the actual best point.

Illustration of the four methods in the simplex method to define new points of simplex is shown in Figure 3-8.

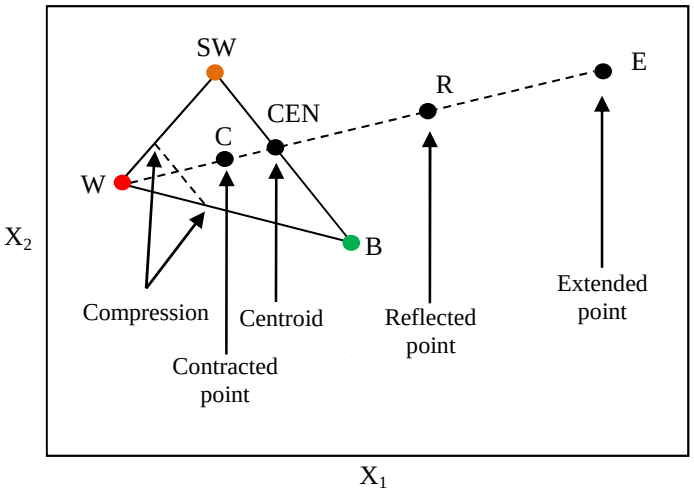

Figure 3-8: Illustration for defining a new simplex by four methods (compression, contraction, reflection and extension) of the simplex method [9].

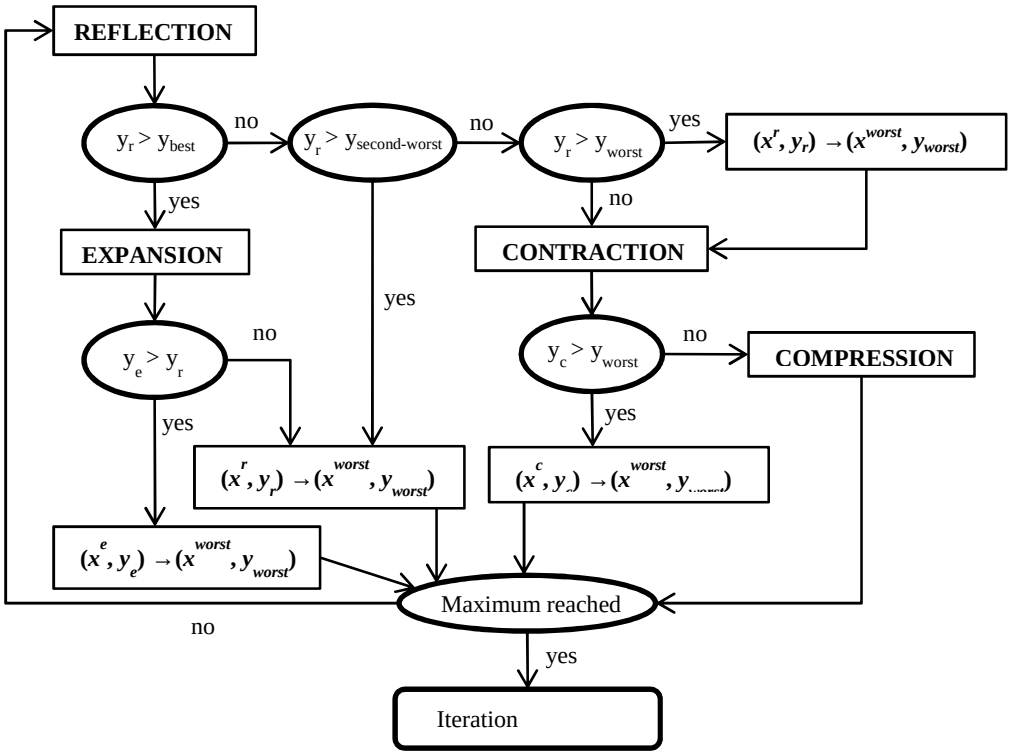

Figure 3-9: Flowchart of the climbing hill algorithm [75].

The evaluation of the response of each observation is shown in Figure 3-9. The algorithm of simplex optimization [9,75] follows these steps:

1. The position of centroid, CEN, which between points B (best response) and SW (second-worst response), is calculated.
2. A reflection of the point W (worst response) is performed through CEN and the response of the point R (reflection) is evaluated. Reflected point, R is assumed within the designated range.
3. If the response of reflected point R is better than best response of point B, this observation indicates that the simplex is moving in the correct direction and it can proceed to step <4> with expansion method. Therefore, an extension of point R, which is twice distance between points CEN and R in the same direction, to point E is tried.
4. Extended point, E is assumed within the designated range. If the response of the extended point E is better than the response of reflected point R, a new simplex

is then formed by replacing W with E, otherwise W is replaced with R. The cycle is repeated from step <1> with the new simplex.

5. If the response of reflected point R is better than second-worst response of point SW but worse than the best response of point B and the worst response of point W, a new simplex is then formed by replacing W with R. The cycle is repeated from step <1> with the new simplex.
6. If the response of reflected point R is better than the worst response of point W but worse than the best response of point B and second-worst response of point SW, a new simplex is then formed by replacing W with R. After that, this can be further proceeded to step <8> with contraction method.
7. If the response of reflected point R is worse than the best response of point B, second-worst response of point SW and the worst response of point W, contraction method is introduced. Please proceed to step <8>.
8. Contracted point C is located at half distance between points CEN and W in the same direction. If the response of the contracted point, C is better than worst response of point, W, a new simplex is then formed by replacing W with C, otherwise, compression method is performed to replace the best response of point B and second-worst response of point SW. The cycle is repeated from step <1> with the new simplex.
9. When no more significant improvement of the response is observed from the moving from one simplex to a new simplex, the iterations can be terminated.

In order to increase the chance of searching multiple optimums and prevent trap in the local extreme, simplex optimization should be performed using 39 different starting points, which consists of 30 randomly selected coordinates and 9 unique design points in the central composite design. In the searching of global extreme, different starting points lead to different optimum within designated range of environmental conditions.

With the sufficient amount of multiple responses, desirability (Equation 3-10) is an efficient utility tool, which ranges from zero (not acceptable) to one (ideal), to optimize multiple responses simultaneously via numerical methods. Besides that, the value of

desirability also evaluates the success rate of the optimized response and experimental configuration from the multiple responses. For desirability, the equation is:

$$D = \sqrt[n]{d_1 x d_2 x \dots x d_n} = \sqrt[n]{\prod_{i=1}^{n} d_i} \quad (3\text{-}10)$$

where *D* and *d* represent the overall desirability of multiple responses and desirability of single response respectively.

Chapter 4 Growth Rate of Chlorella vulgaris

4.1 Introduction

Understanding the growth behaviour of *Chlorella vulgaris* is very important to reduce the relatively high production cost by improving cultivation. From the literature review, it is found that the cultivation of *Chlorella vulgaris* is obviously affected by environmental conditions of bicarbonate concentration, nitrate concentration and light irradiance. Besides that, the interactions between environmental conditions could affect the growth behaviour of *Chlorella vulgaris.* It could hinder the purpose of improving cultivation and achieving designated objective if interactions between environmental conditions are not being considered. The outcome of cultivation could be improved by observing three different responses of growth rate, biomass productivity and lipid productivity with considering the effect of interactions between environmental conditions. With this information, the growth behaviour can be fully understood.

In previous studies [89,90,97,106], the carbon dioxide gas was mainly used to study the kinetic growth of *Chlorella vulgaris*. Due to low solubility of carbon dioxide gas in water, bicarbonate was used instead in this study. To date, there are very few research which are related to the growth rate of *Chlorella vulgaris* under different bicarbonate

concentration. Besides that, nitrate was introduced in this study because nitrogenous element was reported to be able to regulate the cell growth of microalgae. Light irradiance was also taken into consideration since light is one of the crucial photosynthesis elements.

Most literature performed one-factor-at-a-time (OFAT) on the cultivation of microalgae [18]. The critical weakness of OFAT is unable to study the interaction between studied parameters. Therefore, response surface methodology using central composite design was applied to develop experimental configuration for studying the outcome of different level of bicarbonate concentration, nitrate concentration and light irradiance in *Chlorella vulgaris* cultivation. Besides that, multivariate analysis of variance (MANOVA) was applied in the experimental result to investigate the potential of combination of environmental conditions. Three mathematical models will be generated from the central composite design to predict the growth rate, biomass productivity and lipid productivity of *Chlorella vulgaris* with model terms of bicarbonate concentration, nitrate concentration and light irradiance.

The growth behaviour of *Chlorella vulgaris* will be initially discussed in detail based on the responses of growth rate. With the acquired kinetic growth data alone, it contains sufficient information to shorten the cultivation period. Nonetheless, despite that the cultivation period can be shortened, the biomass mass of microalgae could be affected by adjusted environmental conditions. Biomass mass also needs to be taken into the consideration because the heavier mass indicates the better of the harvesting. In order to investigate the biomass production, biomass productivity would be recommended as the response to be observed because biomass productivity is the product of biomass concentration and growth rate.

However, solely improving growth rate and biomass productivity is insufficient to maximise the production rate of algae oil if it is to be used for the production of liquid bio-fuel. Therefore, it is reasonable to move forward to explore the means to increase the lipid inside the microalgae. Hence, increasing lipid within the preferable short time and

high biomass is the upmost important task in this research. Since lipid productivity considers both growth rate and lipid content, the growth behaviour of *Chlorella vulgaris* can be understood more in depth by studying the lipid productivity.

This study mainly focused on kinetic growth, biomass productivity and lipid productivity in microalgae cultivation. Kinetic growth is useful information in microalgae cultivation but it lacks of the biomass and lipid information. On the other hand, biomass productivity contains information of kinetic growth and biomass mass. Optimizing kinetic growth and biomass productivity regression model is not as useful because kinetic growth and biomass productivity do not contain the lipid information. In order to maximize the lipid production of *Chlorella vulgaris* within shorter period of time to lower the production cost, optimization was performed on lipid productivity regression model and discussed in this chapter.

4.2 Single Effect of Medium Composition and Light Irradiance on Chlorella Vulgaris Growth

The cultivation was studied under the designated range of the environmental conditions in factorial design [Figure 2-5 (a)] and axial points of central composite design [Figure 2-5 (b)] explored the middle point and extended points of environmental conditions experiment by extrapolating the designated range. The responses obtained from experiments in this study are presented in the forms of bar charts to investigate single effect of each environmental condition.

4.2.1 Effect of Bicarbonate Concentration on Chlorella Vulgaris Growth

Figure 4-1 illustrates the effect of bicarbonate concentration on the growth rate of *Chlorella vulgaris* under constant nitrate concentration of 1.0 g/L and light irradiance of 4500 LUX. The highest growth rate of *Chlorella vulgaris* was obtained at 5 g/L bicarbonate concentration (4500 LUX and 1 g/L nitrate concentration), while the lowest growth rate was located at 9.2 g/L bicarbonate concentration. It is observed that the

growth rate of *Chlorella vulgaris* increased when the bicarbonate concentration was increased from 0.8 g/L to 5 g/L. Bicarbonate concentration from 0.8 g/L to 5 g/L was assimilated by *Chlorella vulgaris* as carbon source for carbon fixation process.

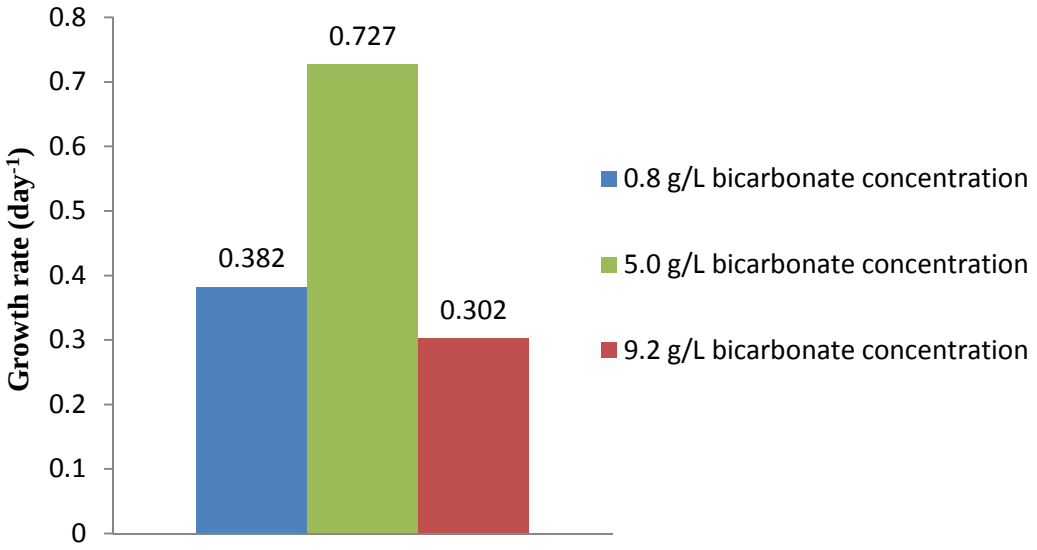

Figure 4-1: Comparison of growth rate of *Chlorella vulgaris* grown in culture medium with different bicarbonate concentration (1 g/L nitrate concentration and 4500 LUX light irradiance)

However, the growth rate dropped down to 0.302 day^{-1} when the bicarbonate concentration was further increased from 5 g/L to 9.2 g/L as shown in Figure 4-1. At beginning of cultivation period of one of the previous research studies [86], *Chlorella pyrenoidosa* demonstrated high photosynthesis efficiency under high concentration of carbon dioxide gaseous when oxygen concentration in medium has not reached saturated level. However, under continuous supply of high concentration of carbon dioxide gaseous, cell had difficulty to transport oxygen from cell to medium due to saturated oxygen concentration in medium. This is supported by Richmond et al. (1982)'s work which mentioned that further increment of bicarbonate to higher concentration led the cultivation to growth inhibition. Besides that, Chen et al. (2010) showed the growth rate of *Chlorella vulgaris* was decreased after the maximum growth rate with further increasing bicarbonate concentration. Therefore, the growth rate of *Chlorella vulgaris* at high bicarbonate concentration of 9.2 g/L was expected to be lower than that of 5 g/L bicarbonate concentration. On the other hand, *Chlorella vulgaris* grown with 0.8 g/L

bicarbonate concentration also had relatively low growth rate because there was lower carbon source supply in culture medium compared to 5 g/L bicarbonate concentration. When the bicarbonate concentration was used up, carbon depletion was initiated and the cell growth was inhibited during cultivation period.

Figure 4-2 shows the biomass productivity of *Chlorella vulgaris* grown in medium with different bicarbonate concentration while the nitrate concentration and light irradiance were fixed at 1 g/L and 4500 LUX respectively.

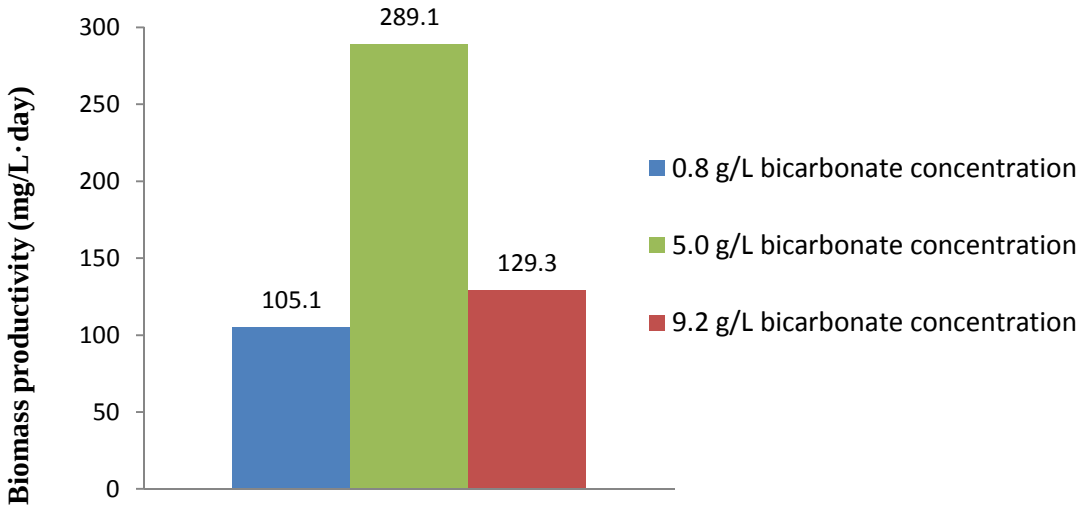

Figure 4-2: Comparison of biomass productivity of *Chlorella vulgaris* grown in culture medium with different bicarbonate concentration (1 g/L nitrate concentration, 4500 LUX light irradiance)

The highest biomass productivity of 289.1 mg/L·day was observed at 5 g/L bicarbonate concentration. It is observed in Figure 4-2 that biomass productivity was decreased when bicarbonate concentration was decreased to 0.8 g/L. Since biomass of *Chlorella vulgaris* is mostly contributed by lipid within microalgae itself, higher bicarbonate concentration is required to produce lipid. 0.8 g/L bicarbonate concentration produced low concentration of carbon dioxide aqueous in the culture medium and there could be insufficient carbon source supply for the biomass production in *Chlorella vulgaris* under these growth conditions (1 g/L nitrate concentration, 4500 LUX light irradiance). As a consequence, this could result in low biomass productivity.

On the other hand, further increment of bicarbonate concentration to 9.2 g/L also decreased the biomass productivity. According to literature findings [89,90,106], *Chlorella* showed the low tolerance over 70% carbon dioxide gas (v/v air) supply. 9.2 g/L bicarbonate might form much too high concentration of carbon dioxide aqueous, which could inhibit the growth of *Chlorella vulgaris*. Similar observation was also observed in Chen et al. (2010)'s experiment. After maximum biomass productivity was reached, further increase in bicarbonate concentration to 9.2 g/L decreased the biomass productivity of *Chlorella vulgaris*. This can be explained by Shelp et al. (1981)'s work that photosynthesis efficiency of *Chlorella pyrenoidosa* is low under high concentration of carbon dioxide gaseous. Low photosynthesis efficiency could result with low biomass production. Hence, the decrement of biomass productivity was expected as the bicarbonate concentration increased.

The lipid productivity of *Chlorella vulgaris* was compared for culture with different bicarbonate concentration (Figure 4-3) when nitrate concentration and light irradiance was fixed at 1 g/L and 4500 LUX respectively.

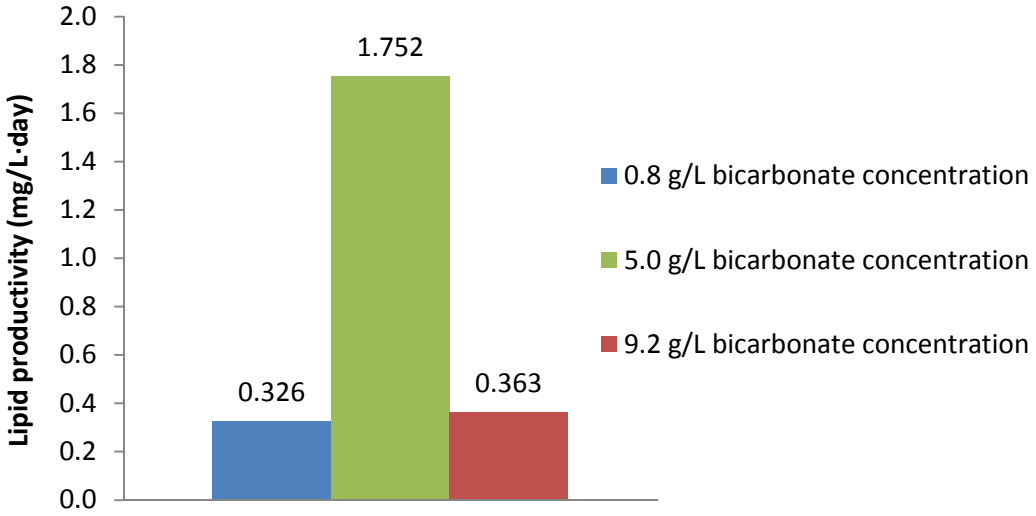

Figure 4-3: Comparison of lipid productivity for *Chlorella vulgaris* grown in culture medium with different bicarbonate concentration (1 g/L nitrate concentration and 4500 LUX light irradiance).

As illustrated in Figure 4-3, the highest value of lipid productivity was obtained for *Chlorella vulgaris* grown in culture with 5 g/L bicarbonate concentration. At 9.2 g/L bicarbonate concentration, the lipid productivity is as low as 0.363 mg/L· day. This could be because 9.2 g/L bicarbonate concentration forms a culture medium with high concentration of carbon dioxide aqueous and this might inhibit the cultivation of *Chlorella vulgaris* due to high carbon dioxide supply. This is supported by conclusion made by Barry et al. (1981) and Sung et al. (1999) that higher carbon dioxide supply will inhibit the growth of *Chlorella*. If the growth of *Chlorella* is inhibited, the biomass productivity and lipid productivity will become relative low because the biomass productivity and lipid productivity is correlated to the growth rate. Besides that, Yeh et al. (2010) also had the same finding that further increment of bicarbonate concentration decreased the lipid content of *Chlorella vulgaris*. Another similar finding shown by Chen et al. (2010) also showed that the lipid content of *Chlorella vulgaris* was decreased as the bicarbonate concentration was increased. If the lipid content of *Chlorella vulgaris* is decreased, the lipid productivity will be lowered. Shelp et al. (1981) also demonstrated that high concentration of carbon dioxide gaseous resulted with low photosynthesis efficiency which could deteriorate lipid production in microalgae cultivation. Therefore, adequate amount of bicarbonate concentration must be carefully taken into consideration in order to prevent growth inhibition and low lipid production of *Chlorella vulgaris*.

On the other hand, as shown in Figure 4-3, the decreasing trend of lipid productivity was found when bicarbonate concentration was decreased from 5 g/L to 0.8 g/L. This is due to the fact that most likely 0.8 g/L bicarbonate concentration formed a culture medium with limited carbon dioxide aqueous. Low concentration of carbon dioxide aqueous could inhibit the lipid accumulation and caused the decrement of lipid productivity. Therefore, in order to boost the lipid productivity, bicarbonate concentration higher than 0.8 g/L is suggested for cultivation of *Chlorella vulgaris*.

4.2.2 Effect of Nitrate Concentration on Chlorella Vulgaris Growth

Figure 4-4 demonstrates the effect of nitrate concentration on the growth rate of *Chlorella vulgaris*. The highest growth rate was observed at 1 g/L nitrate concentration. The growth rate was increased when the nitrate concentration was increased from 0.16 g/L to 1 g/L. However, further increment of nitrate concentration to 1.84 g/L showed the decrement in growth rate.

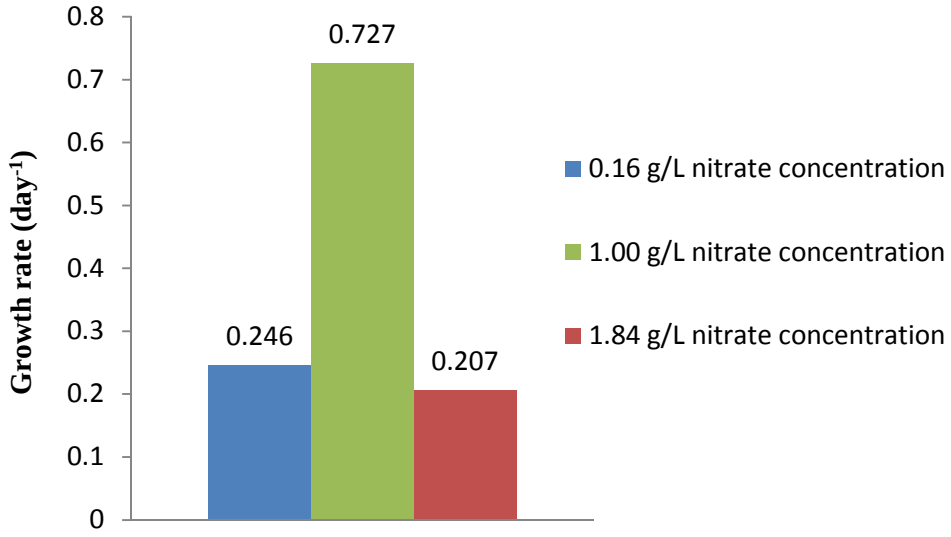

Figure 4-4: Comparison of growth rate of *Chlorella vulgaris* grown in culture medium with different nitrate concentration (5 g/L bicarbonate concentration and 4500 LUX light irradiance).

The lower growth rate of *Chlorella vulgaris* grown in medium with 0.16 g/L nitrate concentration could be due to the lack of nitrogenous element. Nitrogenous element is known to play important role in regulating cell growth of microalgae [54,57,92]. Hence, low nitrate concentration of 0.16 g/L could inhibit the cell growth. This is further supported by findings by Converti et al. (2009), Li et al. (2008) and Chen et al. (2010). Converti et al. (2009) found out that the change in nitrate concentration (from 1.5 g/L to 0.375 g/L) lowered the growth rate of *Chlorella vulgaris* from 0.14 day^{-1} to 0.13 day^{-1}. Besides that, the study done by Li et al. (2008) also showed similar trend, whereby the decrement of nitrate concentration (from 0.850 g/L to 0.255 g/L) decrease the growth

rate of *Chlorella vulgaris* from 0.200 day^{-1} to 0.169 day^{-1}. Chen et al. (2010) also show low growth rate of around 0.4 day^{-1} between 0 g/L to 0.325 g/L nitrate.

On the other hand, the growth rate *Chlorella vulgaris* grown in medium with 1.84 g/L nitrate concentration was reasonably lower than that of 1 g/L. This could be due to extremely high nitrate concentration. When the production rate of ammonia was higher than nitrate assimilation, high nitrate concentration could resulted with ammonia accumulation in cell [17,49]. Uncoupling of phosphorylation lead to adenosine tri-phosphate (ATP) deficiency when excess ammonia is accumulated in cell. ATP deficiency could result with inhibition of carbon fixation process in photosynthesis process. Similar kinetic growth pattern was found by the studies conducted by Chen et al. (2010) and Li et al. (2008). Their study showed that the further increment of nitrate concentration over 0.85 g/L inhibited the growth rate of *Neochloris oleobundans.*

Shown in Figure 4-5 is the biomass productivity of *Chlorella vulgaris* grown in medium with different nitrate concentration when the bicarbonate concentration and light irradiance remained constant.

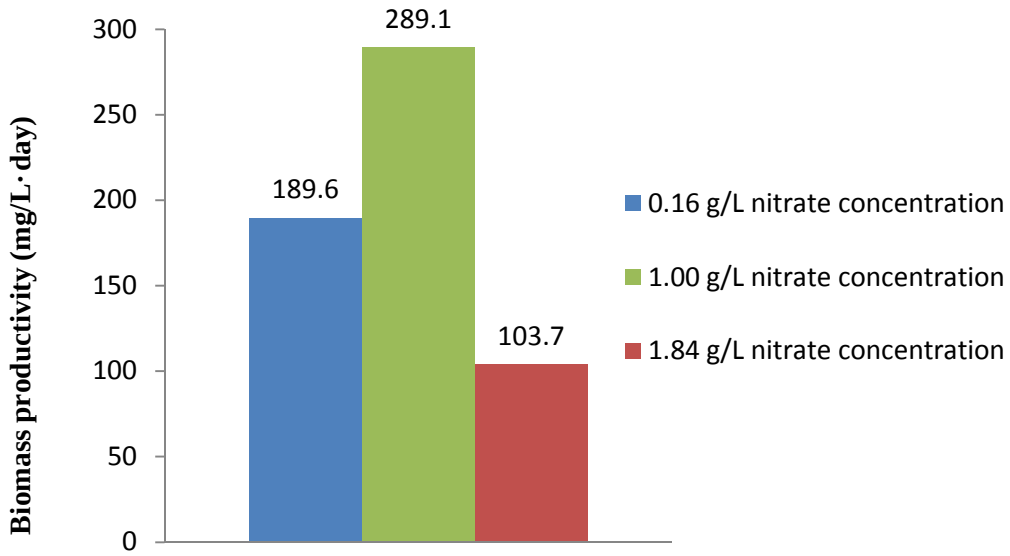

Figure 4-5: Comparison of biomass productivity of *Chlorella vulgaris* grown in culture medium with different nitrate concentration (5 g/L bicarbonate concentration, 4500 LUX light irradiance)

The highest biomass productivity was observed for *Chlorella vulgaris* grown in medium with 1g/L nitrate concentration. When the nitrate concentration was increased from 1 g/L to 1.84 g/L, the biomass productivity was decreased. Same observation which was showed by Li et al. (2008)'s work that the further increment of nitrate concentration to 1.7 g/L decreased the biomass productivity of *Neochloris oleobundas*. This can be supported by Crofts (1966) and Krogmann et al. (1959)'s work that, under excess nitrate concentration, excess ammonia accumulation in cell could resulted with uncoupling of phosphorylation caused ATP deficiency. Hence, biomass production was inhibited by excesss nitrate concentration due to ATP deficiency.

For culture medium with 0.16 g/L nitrate concentration, *Chlorella vulgaris* had biomass productivity of 189.6 mg/L·day. This value was lower that the biomass productivity of microalgae grown in medium with 1 g/L nitrate concentration. It is therefore deduced that nitrate concentration of 0.16 g/L was too low for *Chlorella vulgaris* grown under light irradiance of 4500 LUX.

As shown in Figure 4-6, the lipid productivity of *Chlorella vulgaris* was compared for culture with different concentration of nitrate when the bicarbonate concentration was 5 g/L and light irradiance was 4500 LUX.

The highest value of lipid productivity was achieved for culture medium containing 1 g/L nitrate as shown in Figure 4-6. The lipid productivity decreased when the nitrate concentration was further increased from 1 g/L to 1.84g/L. This pattern is similar to literature finding by Li et al. (2008) and Chen et al. (2010). Chen et al. (2010) showed the highest lipid productivity of *Chlorella vulgaris* at 0.65 g/L nitrate concentration but it decreased with increasing nitrate concentration to 2.6 g/L. Li et al. (2008) also showed the highest lipid productivity at 0.425 g/L nitrate concentration for the *Neochloris oleoabundans* and then followed by the decreasing trend of lipid productivity when nitrate concentration was further increased to 1.7 g/L. In present study, 1.84 g/L nitrate concentration can be considered as excess nitrogen source, which leads to decrement of lipid productivity during cultivation of *Chlorella vulgaris*. Crofts (1966) and Krogmann

et al. (1959)'s explained that ATP deficiency caused by excess nitrate concentration inhibited lipid synthesis process.

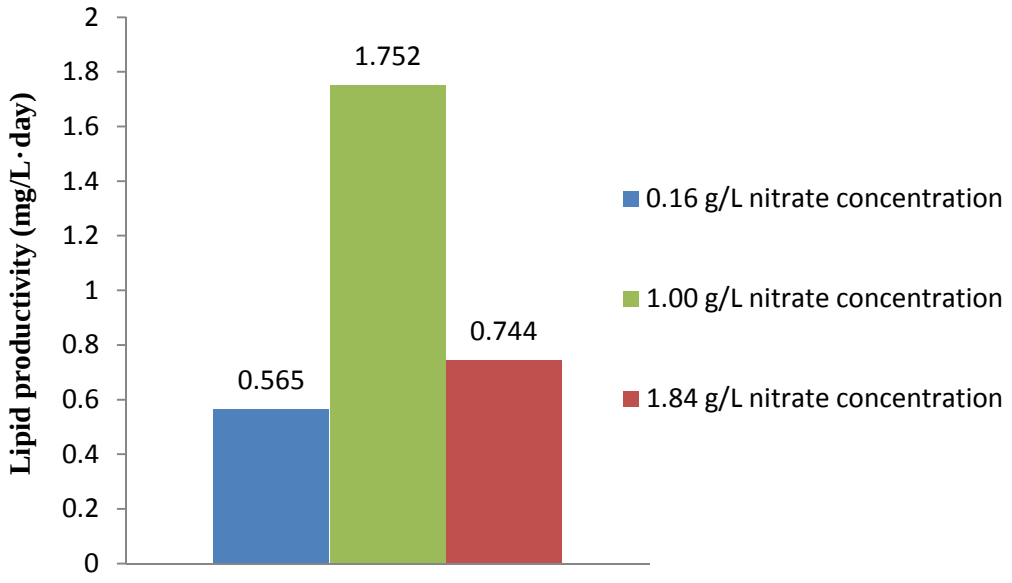

Figure 4-6: Comparison of lipid productivity for *Chlorella vulgaris* grown in culture medium with different nitrate concentration (5 g/L bicarbonate concentration, 4500 LUX light irradiance)

It is observed in Figure 4-6 that the lowest lipid productivity is 0.565 mg/L·day at 0.16 g/L nitrate concentration. According to literature findings [56,59,94], nitrogen element was reported to be the crucial factor to regulate the growth of the microalgae. When the nitrate concentration that is much too low is applied, the growth of *Chlorella vulgaris* will be inhibited, and consequently, lower the lipid productivity.

4.2.3 Effect of Light Irradiance on Chlorella Vulgaris Growth

The growth rate of *Chlorella vulgaris* under different light irradiance is compared in the Figure 4-7 under effects of 5 g/L bicarbonate concentration and light irradiance of 4500 LUX.

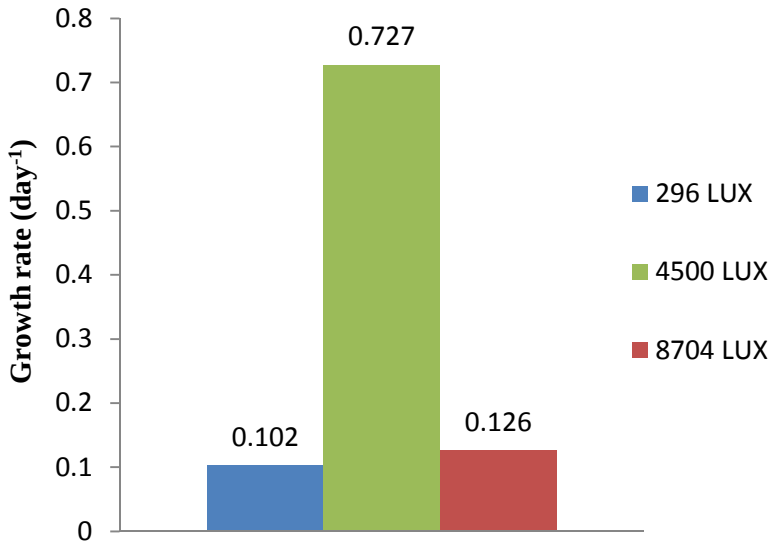

Figure 4-7: Comparison of growth rate of *Chlorella vulgaris* grown in culture medium with light irradiance (5 g/L bicarbonate concentration, light irradiance of 4500 LUX)

The highest growth rate was recorded at 4500 LUX, while extremely low growth rate of *Chlorella vulgaris* was observed at 296 LUX and 8704 LUX. Light irradiance of 296 LUX and 8704 LUX were considered as weak and strong light irradiance respectively. Literature by Carvalho et al. (2011) and Khoeyi et al. (2011) showed that both weak and strong light irradiance can cause the growth inhibition in the cultivation. Therefore, it is deduced that light irradiance of 296 LUX is much too low for the growth of *Chlorella vulgaris* and would cause the photo-deficiency (low light energy), whilst strong light irradiance of 8704 LUX damages the chlorophyll, photosynthesis apparatus and cause the photo-inhibition on the cell growth.

From the comparison of growth rate under different environmental conditions, it is found that excessive or low environmental conditions resulted in unfavoured results. Therefore, adequate amount of environmental conditions should be taken into consideration in order to prevent growth inhibition in cultivation.

Figure 4-8 shows the biomass productivity of *Chlorella vulgaris* grown in medium under different light irradiance when other parameters remained constant.

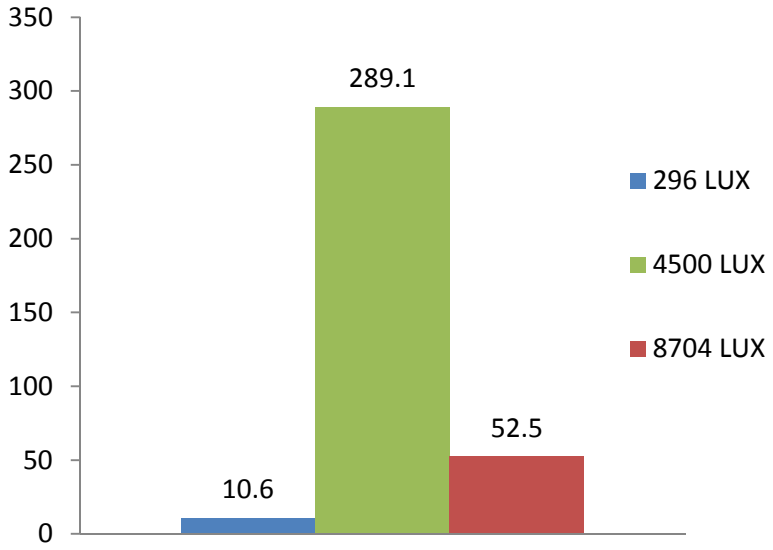

Figure 4-8: Comparison of biomass productivity of *Chlorella vulgaris* grown in culture medium with different light irradiance (5 g/L bicarbonate concentration, 1 g/L nitrate concentration)

As shown in Figure 4-8, at 4500 LUX, the highest biomass productivity was obtained. Light irradiance of 296 LUX could be considered as weak light irradiance while light irradiance of 8704 LUX provided strong light energy to cultivation and damages chlorophyll, photosynthesis apparatus. According to literature evidence [46,81], either strong or weak light irradiance can cause the photo-inhibition to cultivation system and low biomass productivity. This could be the main reason why extremely low biomass productivity of 10.6 mg/L.day and 52.5 mg/L were obtained for culture grown under light irradiance of 296 LUX and 8704 LUX respectively. In other words, for culture with 5 g/L bicarbonate and 1 g/L nitrate concentration, the suitable light irradiance will lie within the range of 296 LUX to 8704 LUX, and likely to be close to 4500LUX. On the other hand, the change in other environmental conditions especially nitrate concentration might have an effect on suitable range of light irradiance for growth of *Chlorella vulgaris.*

Figure 4-9 shows that the comparison of lipid productivity (*Chlorella vulgaris*) under various light irradiance when other parameters remained constant (5 g/L bicarbonate concentration and 1 g/L nitrate concentration).

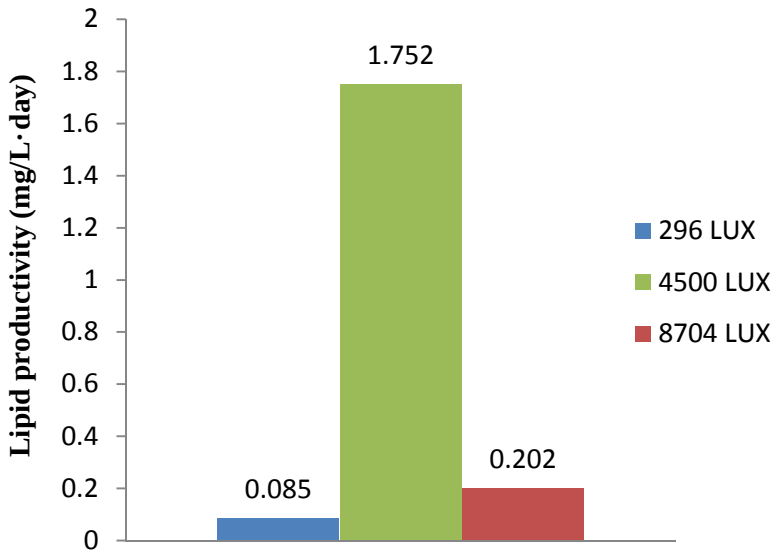

Figure 4-9: Comparison of lipid productivity for *Chlorella vulgaris* grown in culture medium with different nitrate concentration (5 g/L bicarbonate concentration, 1 g/L nitrate concentration)

As shown in Figure 4-9, culture medium grown under light irradiance of 4500 LUX produced the highest lipid productivity. 296 LUX and 8704 LUX showed relative low lipid productivity. As deduced previously, light irradiance of 296 LUX is considered as weak light irradiance. In the photosynthesis, light is an essential element. If weak light irradiance is applied, the photosynthesis process is inhibited and hence the growth rate. As a result, this would lower the lipid productivity due to insufficient light irradiance.

On the other hand, it is deduced that light irradiance of 8704 LUX is too strong and causes photo-inhibition to cultivation for *Chlorella vulgaris* by damaging chlorophyll, photosynthesis apparatus. Therefore, the appropriate amount of supplied light irradiance is required to prevent photo-deficiency or photo-inhibition to microalgae. To stage, light irradiance of 4500 LUX is found to be the optimum intensity that will give the highest lipid productivity.

4.3 Comparison of Experimental Result under Combination of Environmental Conditions

Under the designated range of the environmental conditions, the cultivation was performed according to experimental design developed from central composite design in order to identify interaction between different environmental conditions. In this section, the obtained responses from experiments are presented in the forms of bar charts for the ease of observation on comparison. Valid interaction between environmental conditions on *Chlorella vulgaris* growth will be identified in section 4.4 with using statistical method of multivariate angle of variance (MANOVA).

4.3.1 Comparison of Growth Rate of Chlorella Vulgaris under Combination of Environmental Conditions

The growth rate of *Chlorella vulgaris* grown in culture medium with different combination of environmental conditions at constant nitrate concentration were compared in Figure 4-10. As shown in Figure 4-10 (a), similar growth rate was observed when the bicarbonate concentration was increased for both culture medium under light irradiance of 2000 LUX and 7000 LUX. On the other hand, the increasing trend of cell growth was observed in Figure 4-10 (b) when bicarbonate concentration was increased from 2.5 g/L to 7.5 g/L. Similar observation was found in Yeh et al. (2010) that growth rate of Chlorella vulgaris was decreased when bicarbonate concentrate was increased. The different trend of growth rate observed in Figure 4-10 (a) and Figure 4-10 (b) were mainly due to the varied nitrate concentration in culture medium. This demonstrates the strong relationship between bicarbonate and nitrate concentrations, which affects the growth rate of *Chlorella vulgaris*.

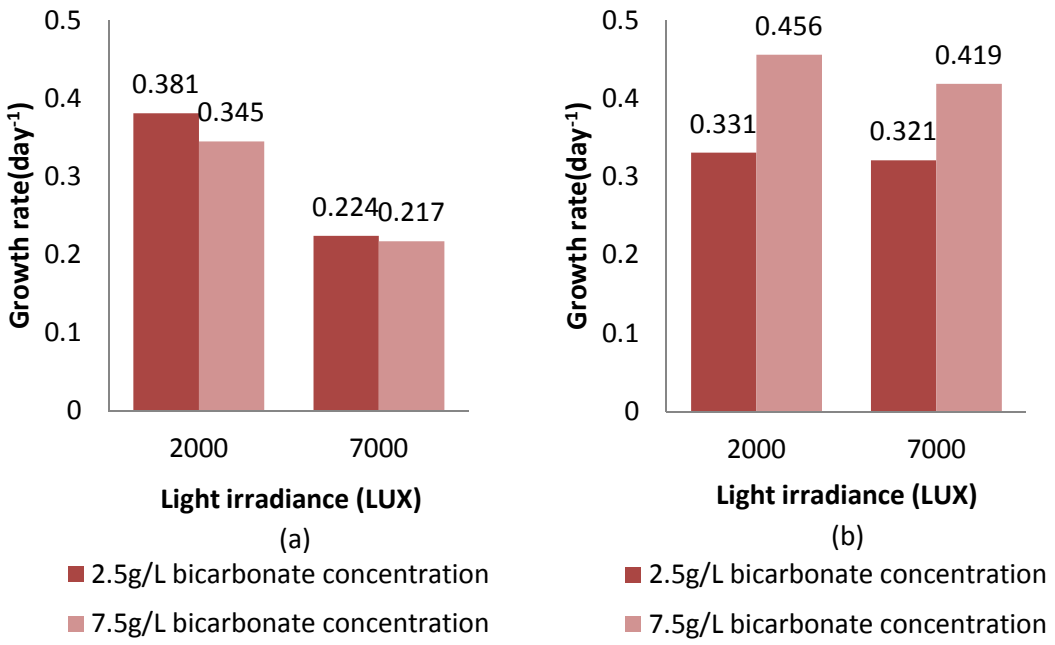

Figure 4-10: Growth rate of *Chlorella vulgaris* grown at constant nitrate concentration (a) 0.5 g/L (b) 1.5 g/L

According to previous studies [16,53,65,94,101], increasing nitrate concentration will increase the cell population. In order to boost cell population, Riebesell et al. (2000) commented that carbon dioxide aqueous is very important to enhance the cell growth. With increasing microalgae, the demand of carbon dioxide aqueous will also increase. Therefore, as shown in Figure 4-10 (b), at higher nitrate concentration, higher demand of bicarbonate concentration was required as the carbon source supply for photosynthesis process so that microalgae can grow faster [42,72,78,79,87,102,103,105].

On the other hand, Figure 4-10 (a) shows that the growth rate was maintained although the bicarbonate concentration was increased. This could be due to the fact that low nitrate concentration (0.5 g/L) could force the cultivation to enter the nitrogen depletion stage and resulted in the decrease in the cell growth. During nitrogen depletion stage, the growth rate of microalgae was inhibited and lipid accumulation began. Although carbon dioxide gas was also reported to have the ability to boost the growth rate of microalgae [89,90,97,106], the effect of nitrate concentration was found to be more substantial compared to carbon dioxide aqueous in cultivation. Hence, it is deduced that increasing

bicarbonate concentration for medium with low nitrate concentration of 0.5 g/L will not aid in enhancing the growth rate.

Displayed in Figure 4-11 is another arrangement for comparison of growth rate under constant bicarbonate concentration. Decreasing growth with increasing light irradiance was observed in Figure 4-11 at 0.5 g/L nitrate concentration. The decrement might be caused by the light irradiance of 7000 LUX. Light irradiance of 7000 LUX may be considered as strong light energy for low cell population and this could cause the photo-inhibition on the cell growth at the beginning of the cultivation [12,46,105].

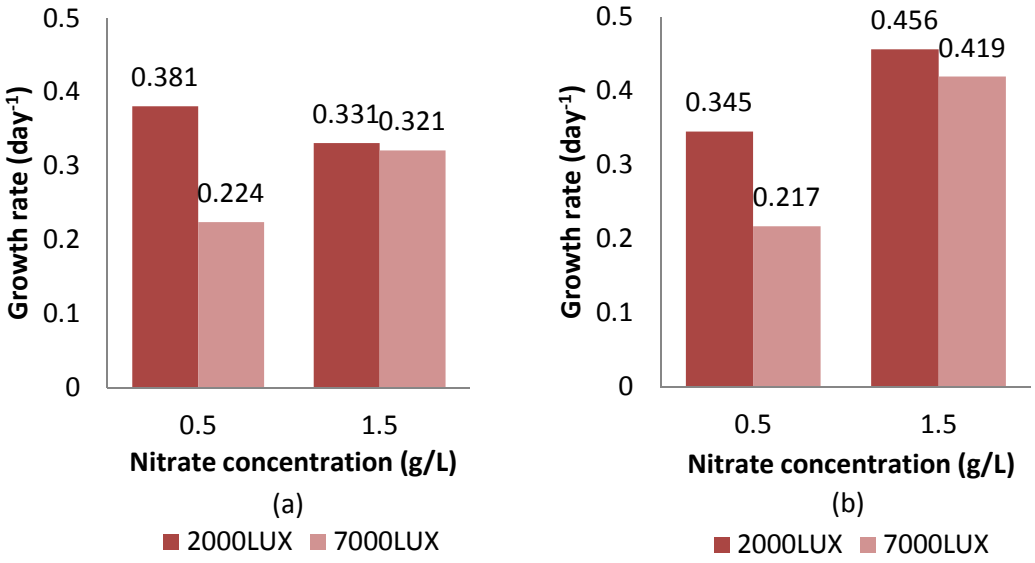

Figure 4-11: Growth rate of *Chlorella vulgaris* grown at constant bicarbonate concentration (a) 2.5 g/L (b) 7.5 g/L

On the other hand, it was observed in Figure 4-11 that at 1.5 g/L nitrate concentration, there was no obvious decrement in growth rate when the light irradiance was set at high light irradiance of 7000 LUX. In other words, *Chlorella vulgaris* grown in culture medium with different nitrate concentration will undergo photo-inhibition at different light irradiance. If referring to results obtained in this study, *Chlorella vulgaris* grown in culture medium with higher nitrate concentration (1.5 g/L) seemed to be able to exhibit high growth rate at high light irradiance. These observations indicated that there were

strong interactions between light irradiance and nitrate concentration, which affected the growth rate of *Chlorella vulgaris.*

Shown in Figure 4-12 is the comparison of growth rate of *Chlorella vulgaris grown* under constant light irradiance. In general, there were increasing trend of growth rate with increasing nitrate concentration. Few researchers reported that nitrate has the potential to regulate the cell growth in the cultivation [16,53,65,94,101]. As illustrated in Figure 4-12, the growth rate was boosted by increasing nitrate concentration under the supply of bicarbonate and light irradiance. It shows that the nitrate concentration is a very important environmental condition in cultivation of *Chlorella vulgaris*.

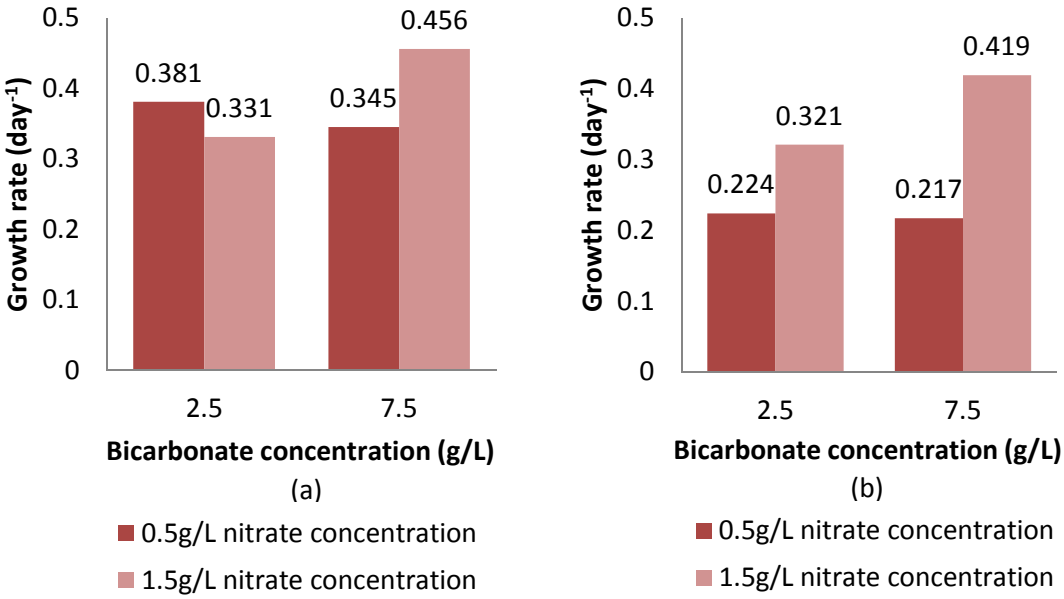

Figure 4-12: Growth rate of *Chlorella vulgaris* grown at constant light irradiance (a) 2000 LUX (b) 7000 LUX

On the other hand, Figure 4-12 (a) shows that the change of growth rate was almost negligible when the nitrate concentration was increased for culture medium with 2.5 g/L bicarbonate concentration at light irradiance of 2000 LUX. This could be explained by the fact that photo-deficiency or carbon depletion could happen when *Chlorella vulgaris* were grown under these conditions. Although higher nitrate concentration would boost

the cell population, the ability of light to penetrate culture medium would be reduced and less light energy would be available in culture when cell population got higher.

In summary, it is found that nitrate concentration in culture medium has strong effect on the growth rate of *Chlorella vulgaris.* It is also observed that higher bicarbonate concentration that promotes higher growth rate of *Chlorella vulgaris* is found to be dependent on the amount of nitrate concentration in culture medium. The observations also indicated that there were strong correlation between light irradiance and nitrate concentration, which affected the growth rate of *Chlorella vulgaris.* It is concluded that all the three environmental conditions (nitrate concentration, bicarbonate concentration and light irradiance) are closely correlated to one another in affecting the growth rate of *Chlorella vulgaris*.

4.3.2 Comparison of Biomass Productivity of Chlorella Vulgaris under Combination of Environmental Conditions

Figure 4-13 displays the biomass productivity of *Chlorella vulgaris* grown in medium with similar nitrate concentration. As shown in Figure 4-13 (b), when the bicarbonate concentration was increased from 2.5 g/L to 7.5 g/L, similar biomass productivity was found for culture grown at 2000 LUX. On the other hand, *Chlorella vulgaris* grown in other culture medium (shown in both Figure 4-13(a) and (b)) showed significant decrease in biomass productivities when bicarbonate concentration was increased. However, as shown in Figure 4-10 (b), it is observed that increase in bicarbonate concentration enhanced growth rate of *Chlorella vulgaris*. This is supported by findings of Yeh et al. (2010) who mentioned that further increment of bicarbonate concentration improved growth rate of *Chlorella vulgaris* but carbon source utilization for biomass production was gradually decreased. Hence, decrease in carbon source utilization could lower the rate of biomass production although high bicarbonate concentration promoted growth rate of *Chlorella vulgaris*.

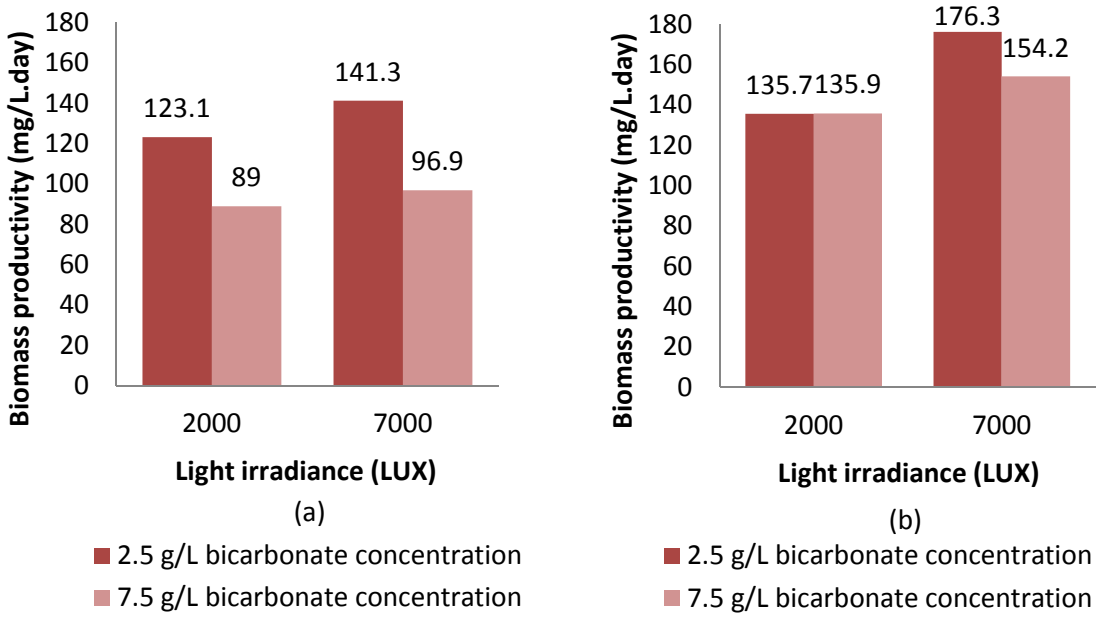

Figure 4-13: Biomass productivity of *Chlorella vulgaris* grown at constant nitrate concentration of (a) 0.5 g/L (b) 1.5 g/L

The comparison of biomass productivity of (*Chlorella vulgaris*) grown at constant light irradiance is displayed in Figure 4-14. It is observed in Figure 4-14 (a) and (b) that the increment of nitrate concentration from 0.5 g/L to 1.5 g/L significantly increased the biomass productivity of *Chlorella vulgaris* when light irradiance and bicarbonate concentration remained constant. This increasing trend was supported with literature findings by Li et al. (2008) and Li et al. (2011). Li et al. (2008) found that there is increment in biomass productivity of microalgae with increasing nitrate concentration. On the other hand, the study by Li et al. (2011) demonstrated that the maximum of biomass productivity (*Chlorella minutissima*) was located between 1.56 g/L and 2.34 g/L nitrogen concentration.

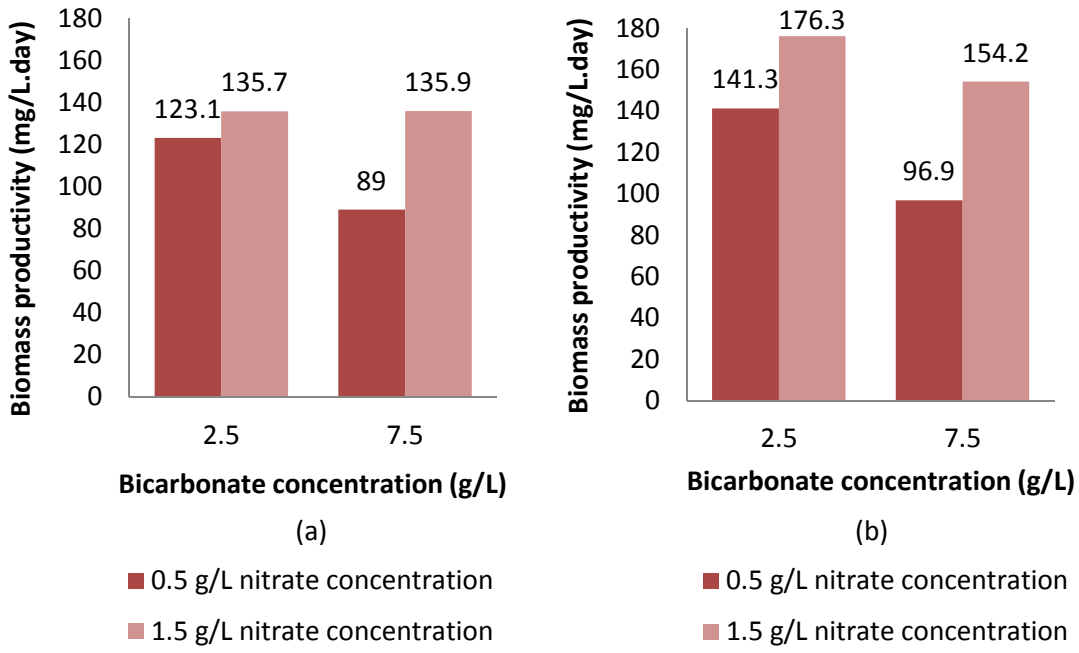

Figure 4-14: Biomass productivity of *Chlorella vulgaris* grown at constant light irradiance of (a) 2000 LUX (b) 7000 LUX

As shown in Figure 4-15, the biomass productivity of *Chlorella vulgaris* grown under different growth conditions at constant light irradiance was compared. In contrast to the trend shown for the growth rate, biomass productivity of *Chlorella vulgaris* was increased when light irradiance was increased from 2000 LUX to 7000 LUX.

Hence, it is deduced that significant effect of light irradiance on biomass productivity was identified. The increasing biomass productivity with increasing light irradiance agrees with previous findings by Khoeyi et al. (2007) and Sandnes et al. (2005). Khoeyi et al. (2007) showed the biomass productivity of *Chlorella vulgaris* was increased from 0.65 g/L.day to 2.32 g/L.day when the light irradiance was increased from 2775 LUX to 7400 LUX. On the other hand, Yeh et al. (2010) also demonstrated that higher light irradiance increased the carbon source utilisation rate.

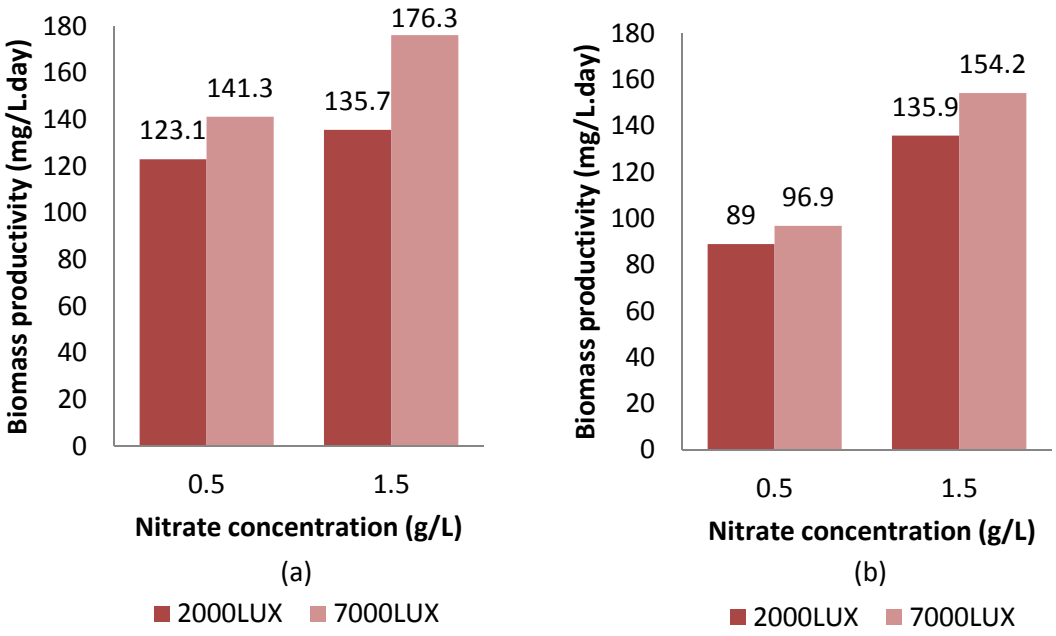

Figure 4-15: Biomass productivity of *Chlorella vulgaris* grown at constant bicarbonate concentration of (a) 2.5 g/L (b) 7.5 g/L

Interaction was also observed between the nitrate concentration and light irradiance. As shown in Figure 4-15, higher nitrate concentration was able to boost the biomass productivity when the light irradiance increased from 2000 LUX to 7000 LUX. This showed that the higher light irradiance and higher nitrate concentration favour the biomass productivity of *Chlorella vulgaris*.

Nitrate concentration and light irradiance shows the positive effect in cultivation of *Chlorella vulgaris*. Although higher bicarbonate concentration deteriorates the biomass productivity, adequate bicarbonate concentration is necessary for the cell growth.

4.3.3 Comparison of Lipid Productivity of Chlorella Vulgaris under Combination of Environmental Conditions

Shown in Figure 4-16 is the comparison of lipid productivity of *Chlorella vulgaris* grown at constant nitrate concentration of 0.5 g/L and 1.5 g/L. It is observed in the figure that generally, there is increasing trend with increased bicarbonate concentration except for culture with 1.5 g/L nitrate concentration at light irradiance of 2000 LUX.

However, as shown in Figure 4-10, growth rate of *Chlorella vulgaris* at low nitrate concentration of 0.5g/L was maintained although bicarbonate concentration was increased. Widjaja et al. (2009) commented that *Chlorella vulgaris* cultivation exposing to nitrogen starvation resulted in the increase in lipid content. As nitrogen assimilation was gradually inhibited due to low nitrate concentration, adenosine tri-phosphate (ATP) for nitrogen assimilation was shifted to support catalytic process in lipid synthesis pathway required a lot of energy derived from ATP [15]. Lipid accumulation was enhanced when nitrogen source was gradually depleted. Hence, this shows that bicarbonate concentration contributes in improving the lipid productivity of *Chlorella vulgaris.*

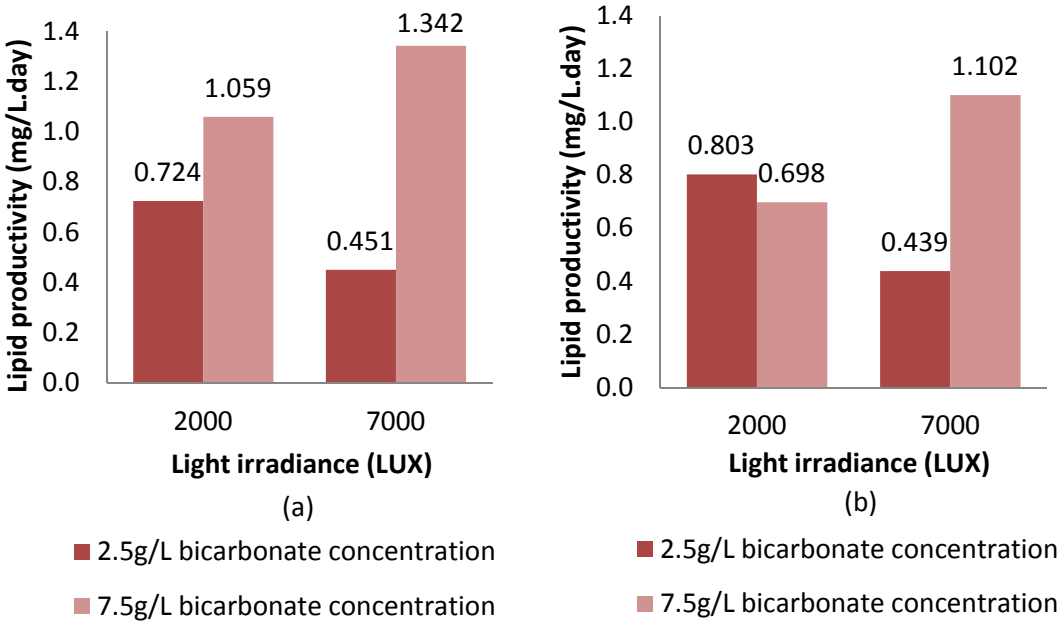

Figure 4-16: Lipid productivity of *Chlorella vulgaris* grown at constant nitrate concentration (a) 0.5 g/L (b) 1.5 g/L

On the other hand, for culture with higher nitrate concentration (1.5 g/L) at light irradiance of 2000 LUX, there is slight decrease in lipid productivity with increasing bicarbonate concentration. *Chlorella vulgaris* cultivation under high nitrate concentration could promote cell population growth of *Chlorella vulgaris*. However, high cell population needed longer time in competitively absorbing weak light irradiance of 2000 LUX for the synthesis of ATP. Low ATP production could inhibit lipid

production pathway which required a lot of energy derived from ATP. In fact, the increase in lipid productivity for culture grown in 0.5 g/L nitrate is also less significant at low light irradiance. Low nitrate concentration of 0.5 g/L limited cell population growth of *Chlorella vuglaris*. Low cell population can absorb weak light irradiance without having competitive in absorbing light. This explained lipid accumulation was enhanced under nitrate depletion. It is therefore implied that the effect of bicarbonate on lipid productivity is dependent on the range of light irradiance used for cultivation.

Figure 4-17 shows the comparison of lipid productivity of *Chlorella vulgaris* grown at light irradiance of 2000 LUX and 7000 LUX. As shown in Figure 4-17, at 2.5 g/L bicarbonate concentration, there was neither significant increment nor decrement in the lipid productivity when nitrate concentration was increased for both culture grown under 2000 LUX and 7000 LUX. This shows that the effect of nitrate concentration on lipid productivity of *Chlorella vulgaris* is insignificant at low bicarbonate concentration of 2.5 g/L.

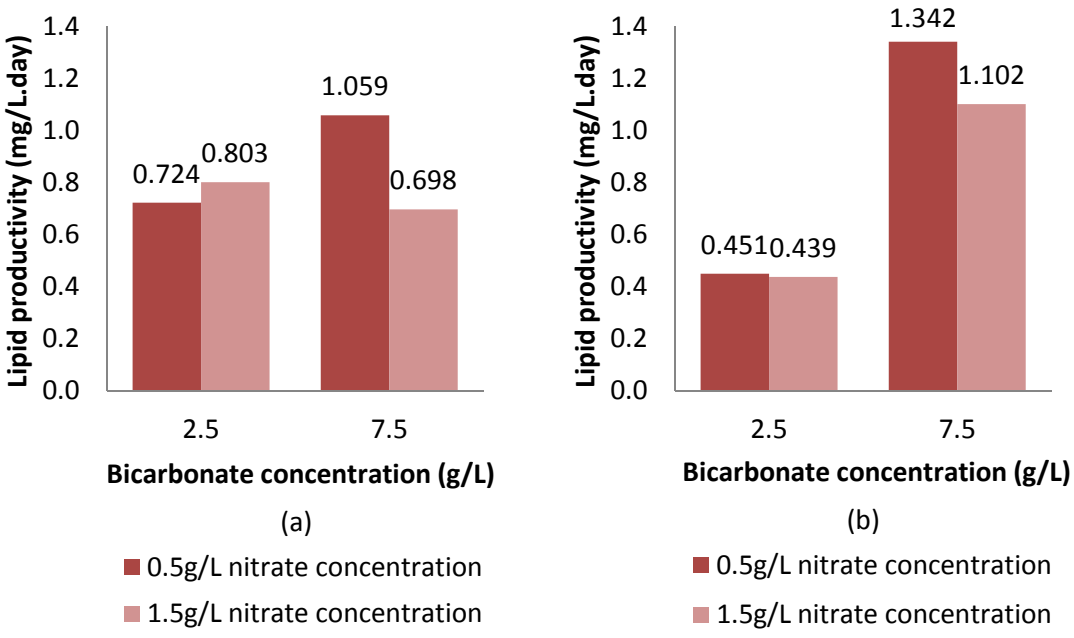

Figure 4-17: Lipid productivity of *Chlorella vulgaris* grown at constant light irradiance (a) 2000 LUX (b) 7000 LUX

More significant decreasing trend of lipid productivity, however, was noticed at 7.5 g/L bicarbonate concentration when nitrate concentration was increased (Figure 4-17). The decreasing trend of lipid productivity (Figure 4-17) was similar to the studies by Li et al. (2008), Chen et al. (2010) and Converti et al. (2009), whereby further increment of nitrogen concentration decreased the lipid productivity.

Nitrogen source was reported to be able to regulate the growth of *Chlorella vulgaris*. Under normal growth environment, the *Chlorella vulgaris* will eventually grow into matured cell, which will then perform the mitosis process to split into two identical cells. During this stage, cell population is increased but lipid content is divided equally between two cells. Lipid accumulation is unlikely to happen in this stage due to mitosis process. This is the reason why lower lipid productivity were obtained for *Chlorella vulgaris* grown in culture with higher nitrate concentration of 1.5 g/L as it promotes higher cell population. Furthermore, the increase in the growth rate of *Chlorella vulgaris* with increasing nitrate concentration (under the same growth conditions) is supported by previous findings in section 4.3.1.

To confirm the presumption that lower lipid productivity was contributed by lower lipid content instead of lower cell population, lipid content of *Chlorella vulgaris* grown under these conditions was examined in Figure 4-18. For culture medium with 2.5 g/L bicarbonate concentration, the change of lipid content of *Chlorella vulgaris* was negligible as the nitrate concentration increased. On the other hand, there is decreasing lipid content of *Chlorella vulgaris* with increasing nitrate concentration for culture medium with 7.5 g/L bicarbonate. This further affirms the inference that the lower productivity in this case is a result of the reduction in lipid accumulation.

From the findings above, it is deduced that higher nitrate concentration is not favourable in enhancing the lipid productivity. Therefore, in enhancing lipid productivity of *Chlorella vulgaris through* the lipid accumulation, low nitrate concentration will be recommended instead. Also, it should be noted that the contribution of lower nitrate concentration to higher lipid productivity will be dependent on the amount of

bicarbonate concentration. Therefore, nitrate and bicarbonate concentration are correlated in affecting the lipid productivity of *Chlorella vulgaris.*

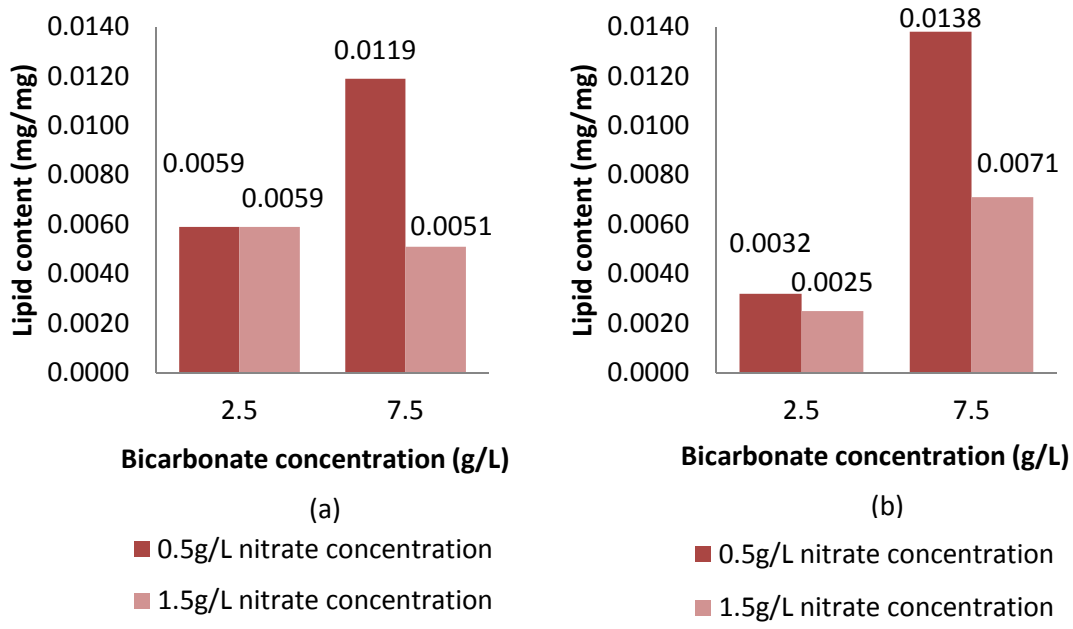

Figure 4-18: Lipid content of *Chlorella vulgaris* grown at constant light irradiance (a) 2000 LUX (b) 7000 LUX

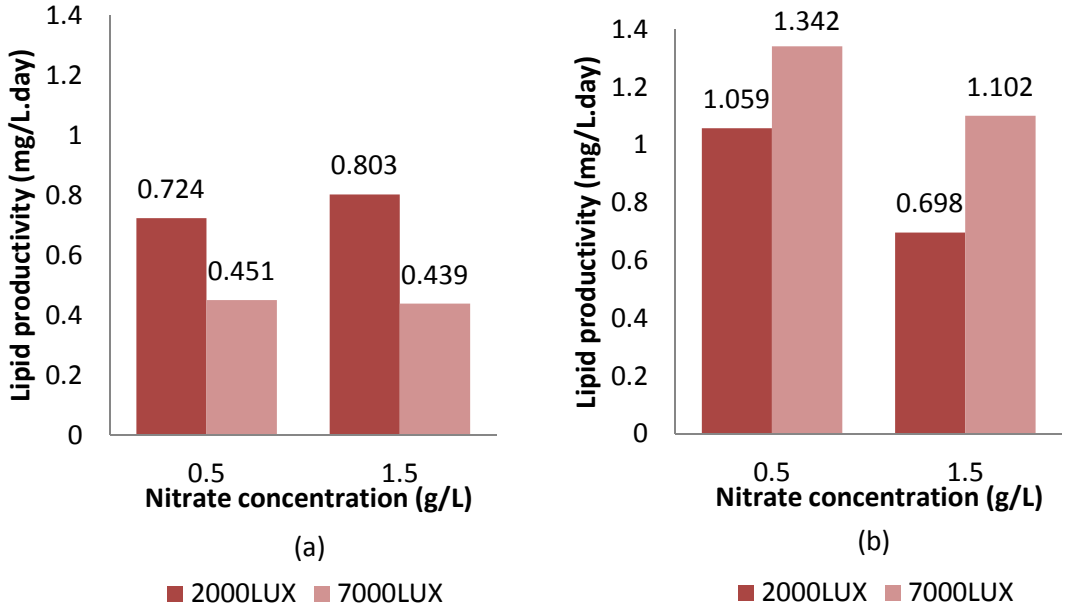

Figure 4-19: Lipid productivity of *Chlorella vulgaris* grown at constant bicarbonate concentration (a) 2.5 g/L (b) 7.5 g/L

Presented in Figure 4-19 is another comparison of lipid productivity for *Chlorella vulgaris* grown in medium with constant bicarbonate concentration. As presented in Figure 4-19 (a), decreasing trend of lipid productivity was noticed with increment of the light irradiance for culture with 2.5 g/L bicarbonate concentration. Yeh et al. (2010) demonstrated that increasing light irradiance will result in higher carbon source utilisation and decrease in growth rate. Since low bicarbonate concentration of 2.5 g/L was introduced, this would form low carbon source supply in culture system. Low amount of bicarbonate would have been used up in shorter period of time and *Chlorella vulgaris* will no longer able to perform lipid accumulation without carbon source. This explains the decrement trend of lipid productivity, as illustrated in Figure 4-19 (a). Furthermore, as the light irradiance was increased, photo inhibition could have occurred. The cell growth would then be inhibited, which will contribute further to lower lipid productivity, increase in light irradiance decreased growth rate of *Chlorella vulgaris*, as shown in Figure 4-11.

On the other hand, opposite trend was observed for culture with 7.5 g/L bicarbonate concentration (Figure 4-19 (b)), whereby the lipid productivity increased with increasing light irradiance. This is mainly because the carbon source utilisation is increased when light irradiance is increased. Since the lipid consists of carbon and hydrogen elements, higher demand of carbon element would be expected with increasing light irradiance. Besides that, the carbon source demand was also increased due to the increase in cell population with increasing nitrate concentration. Therefore, there is great demand for the carbon source. When high bicarbonate concentration of 7.5 g/L was introduced, high carbon source supply would be formed in the culture medium. This would overcome the great demand of carbon source supply and promote the lipid accumulation with higher lipid productivity of microalgae. Besides that, light irradiance also provides necessity energy for the microalgae to assimilate carbon dioxide aqueous. Therefore, the increasing trend of lipid productivity demonstrated that higher bicarbonate concentration is able to stimulate much higher lipid productivity at high light irradiance of 7000 LUX.

The above outcomes shows that light irradiance could have strong interaction with bicarbonate concentration, which affects the lipid productivity of *Chlorella vulgaris*. It is concluded that suitable range of light irradiance for maximum lipid productivity of *Chlorella vulgaris* will be dependent on the amount of bicarbonate or carbon source in culture medium.

In this study, bicarbonate concentration showed important contribution in lipid accumulation because lipid is mainly made up of carbon and hydrogen elements. During lipid accumulation, higher light irradiance is favourable for higher bicarbonate concentration because the light irradiance boost the carbon source utilisation in the culture medium. On the other hand, lower nitrate concentration is preferable to improve lipid productivity because nitrogen depletion can enhance the lipid accumulation within the cell. It is also noticed that all the environmental conditions are correlated to one another in affecting the lipid productivity.

4.4 Multivariate Analysis of Variance on Chlorella Vulgaris Growth

Multivariate analysis of variance (MANOVA) was applied on experimental result obtained from central composite design experimental design. Three mathematical models were developed to illustrate growth rate, biomass productivity and lipid productivity of *Chlorella vulgaris* under effect of bicarbonate concentration, nitrate concentration and light irradiance. From MANOVA result, model fitness can be justified and interaction between environmental conditions can be identified.

4.4.1 Growth Rate of Chlorella Vulgaris

Figure 4-20 and Figure 4-21 show the normal plot of residuals and scatter plot of residuals versus predicted values of growth rate (Central composite design) respectively. The position of residuals that is shown in Figure 4-20 was close to the straight line of normality. On the other hand, the residuals, which are shown in Figure 4-21, were evenly scattered and not close to each other. These observations show that the residuals

did not exhibit any non-constant variance or non-normality behaviour and no response transformation is required.

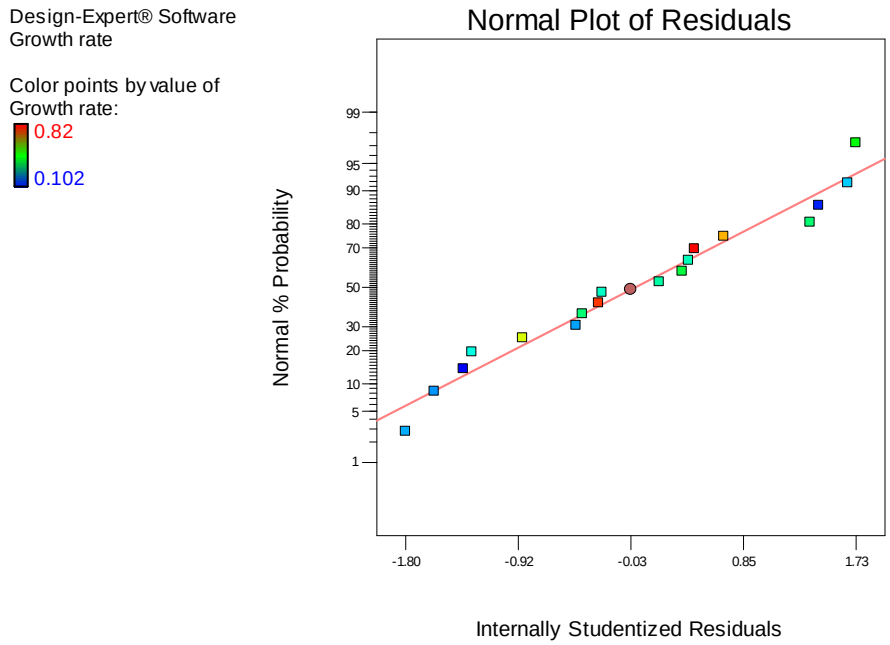

Figure 4-20: Normal plot of residuals of growth rate (Central composite design)

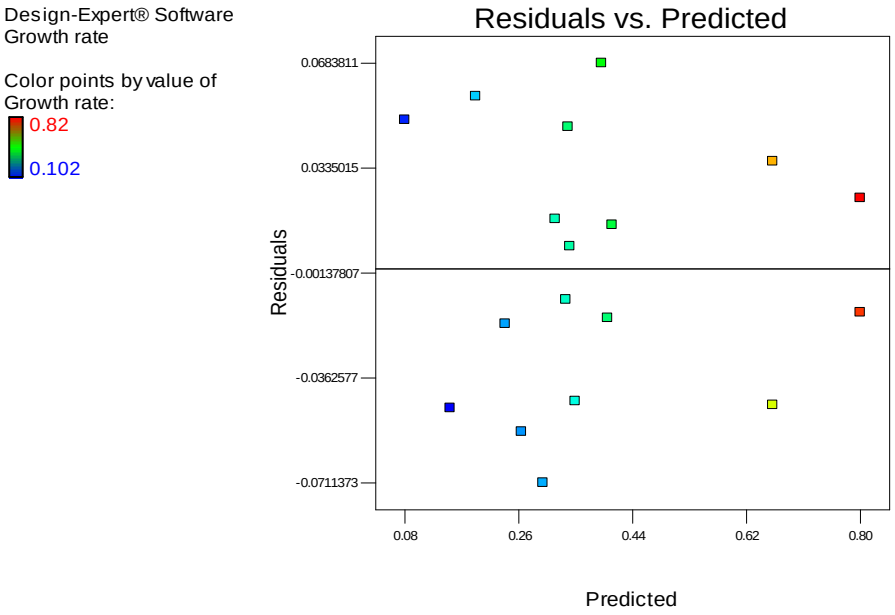

Figure 4-21: Scatter plot of residuals versus predicted value of growth rate (Central composite design)

A mathematical model for growth rate fitted in terms of actual factors is shown as follow:

$$Growth\ rate = -0.73161 + 0.15455A + 1.0288B + 2.45576x10^{-4}C + 0.0266AB + 2.38x10^{-5}BC - 0.017982A^2 - 0.61289B^2 - 3.08797x10^{-8}C^2 \quad (4\text{-}1)$$

The results of multivariate of variance of analysis (MANOVA) are shown in Table 4-1 . The A, B and C, which are shown in Table 4-1, represent bicarbonate concentration, nitrate concentration and light irradiance respectively. The fitness of secondary order polynomial model with all model terms (A, B, C, AB, AC, BC, A^2, B^2, C^2 and ABC) falls below satisfactory level. It is necessary to omit model terms with higher probability value from the model in order to improve the fitness of the developed model. Hence, model terms of AC and ABC are omitted from growth rate mathematical model since probability value for model terms of AC and ABC are closed to 1.

Table 4-1: Multivariate analysis of variance for growth rate (Central composite design)

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	Significance
Block	0.041	1	0.041			
Model	0.7	8	0.087	23.21	< 0.0001	significant
A-Bicarbonate	1.513E-04	1	1.5130E-04	0.04	0.8460	
B-Nitrate	6.347E-03	1	6.3470E-03	1.69	0.2299	
C-Light Irradiance	6.228E-03	1	6.2280E-03	1.66	0.2339	
AB	8.845E-03	1	8.8450E-03	2.35	0.1635	
BC	7.081E-03	1	7.0810E-03	1.88	0.2071	
A^2	0.16	1	0.16	42.26	0.0002	
B^2	0.3000	1	0.3000	78.55	< 0.0001	
C^2	0.4700	1	0.4700	124.63	< 0.0001	
Residual	0.0300	8	0.0038			
Lack of Fit	0.0260	6	0.0043	2.17	0.3486	not significant
Pure Error	0.0040	2	0.0020			
Cor Total	0.7700	17				
R-Squared	95.87%					

In Table 4-1, significant threshold is set at 0.20. Significant Model is supported by probability value of <0.0001, which is below significance threshold of 0.20. The value of the coefficient of the multiple determination, R-squared value of 95.87%, could also explain the significance of model fitness. Besides that, Lack of Fit with probability value of 0.3486 indicates that the model is not significant relative to the pure error.

It is clearly observed in Table 4-1 that A (probability value of 0.8460) is not important model term. Although the probability values of B (0.2239), C (0.2339) and BC (0.2071) are just barely above than significance threshold of 0.20, model terms of B, C and BC can easily pass the significance test. Therefore, the valid model terms are B, C, AB (probability value of 0.1635), BC, A^2 (probability value of 0.0002), B^2 (probability value of <0.0001) and C^2 (probability value of <0.0001).

Deduced from the MANOVA results (Table 4-1), as indicated by the smaller probability value (~0.0001), bicarbonate concentration (A^2), nitrate concentration (B^2) and light irradiance (C^2) individually contribute the non-linearity effect in cell growth of *Chlorella vulgaris*. The small probability value of 0.1635 also implies that combination of nitrate and bicarbonate concentrations (AB) also contributes strong effect on the cell growth during cultivation. As remarked by high probability value (~0.22), nitrate concentration (B) and light irradiance (C) individually produce the least contribution in the cell growth. Besides that, the strong interaction is demonstrated by the combination of nitrate concentration and light irradiance (BC) in cell growth of *Chlorella vulgaris*. These observations are also consistent with the observations in section 4.3.1.

MANOVA result on growth rate indicates that the bicarbonate concentration, nitrate concentration and light irradiance are great important environmental conditions in cell growth of *Chlorella vulgaris*. Besides that, both combination of environmental conditions (bicarbonate and nitrate concentration) and (nitrate concentration and light irradiance) are able to enhance the cell growth of *Chlorella vulgaris*.

4.4.2 *Biomass Productivity of Chlorella Vulgaris*

The mathematical model for biomass productivity (Equation 4-2) was built fitted in terms of actual factors.

$$Biomasss\ productivity = -345.08013 + 79.92475A + 296.71355B + 0.1241C + 5.66\,AB - 6.52x10^{-4}AC + 3.28x10^{-3}BC - 8.43993A^2 - 163.34975B^2 - 1.32850x10^{-5}C^2 \quad (4\text{-}2)$$

Figure 4-22 and Figure 4-23 show normal plot of residuals and scatter plot of residuals versus predicted of biomass productivity (Central composite design) respectively. Figure 4-22 shows that the residuals were close to the straight line of normality while Figure 4-23 shows that residuals scattered in the plot. These observations indicated that residuals supported the multivariate analysis of variance (MANOVA) assumptions of normality and constant variance and hence, no response transformation is applied.

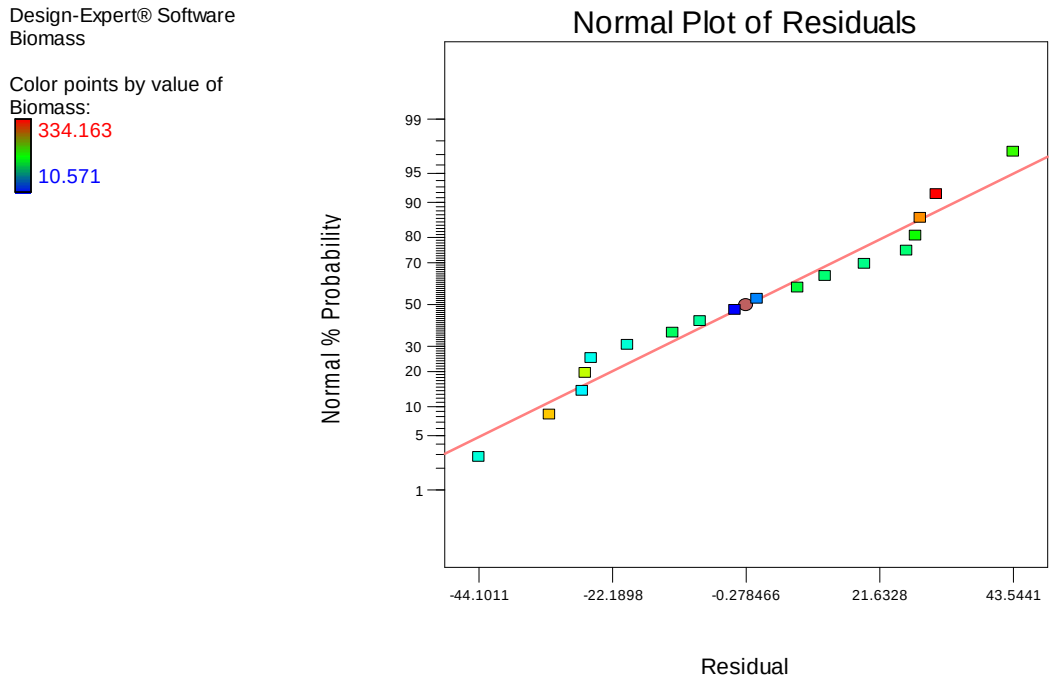

Figure 4-22: Normal plot of residuals of biomass productivity (Central composite design)

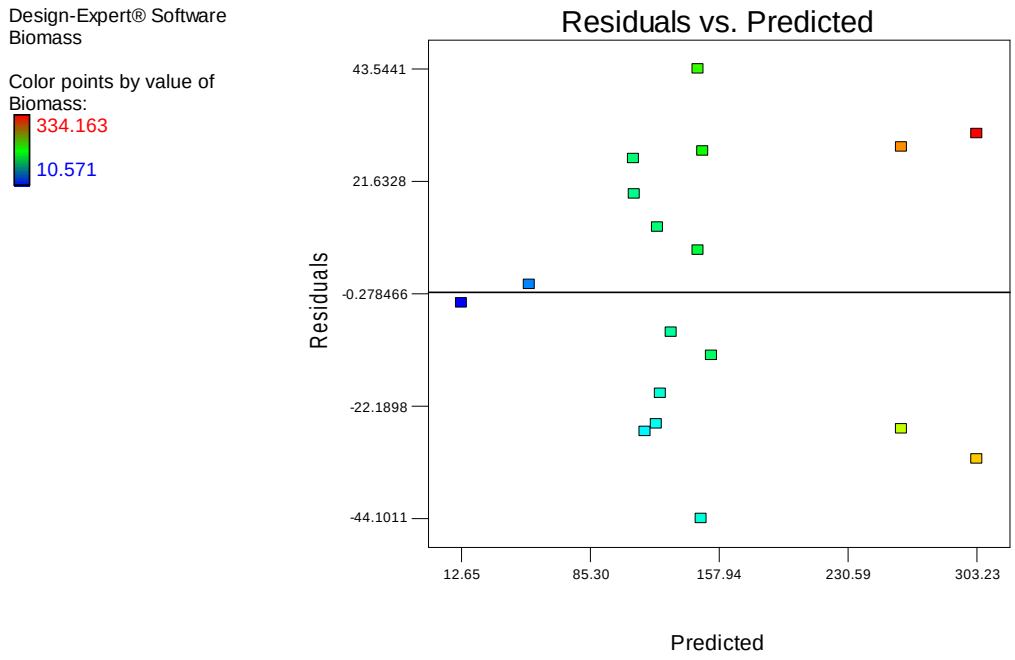

Figure 4-23: Scatter plot of residuals versus predicted value of biomass productivity (Central composite design)

Table 4-2: Multivariate analysis of variance for biomass productivity (Central composite design)

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	Significance
Block	1554.18	1	1554.18			
Model	110100.00	9	12229.64	8.01	0.0060	significant
A-Bicarbonate	260.98	1	260.98	0.17	0.6916	
B-Nitrate	3.94	1	3.94	0.00	0.9609	
C-Light Irradiance	1769.81	1	1769.81	1.16	0.3173	
AB	400.45	1	400.45	0.26	0.6243	
AC	132.84	1	132.84	0.09	0.7765	
BC	134.48	1	134.48	0.09	0.7752	
A^2	34980.24	1	34980.24	22.92	0.0020	
B^2	22533.77	1	22533.77	14.76	0.0064	
C^2	86670.18	1	86670.18	56.79	0.0001	
Residual	10683.41	7	1526.20			
Lack of Fit	8858.98	5	1771.80	1.94	0.3738	not significant
Pure Error	1824.42	2	912.21			
Cor Total	122300.00	17				
R-Squared	91.15%					

The result of MANOVA is shown in Table 4-2 where A, B and C represent bicarbonate concentration, nitrate concentration and light irradiance respectively. Since the fitness of biomass productivity model does not meet satisfactory level with model terms of A, B, C, AB, AC, BC, A^2, B^2, C^2 and ABC, model term with high probability value is necessary to be omitted. Although the probability value for most of the model terms in biomass productivity model is very high, it is acceptable to keep model terms with high probability value to maintain the originality of mathematical model. Hence, model terms of ABC, with the highest probability value, is omitted from biomass productivity model.

Significant threshold is set at 0.30 in Table 4-2. The Model (probability value of 0.006) implies that the model is significant and non-significance of Lack of Fit is supported by probability value of 0.3738. Besides that, R-squared value of 91.15% shows that the model fits data well.

As presented in Table 4-2, model term of ABC with high probability value was removed from model. On the other hand, the valid terms are model terms A^2 (probability value of 0.0020), B^2 (probability value of 0.0064) and C^2 (probability value of 0.0001). Since model terms of A^2, B^2 and C^2 are valid model terms, the model terms of A and B cannot be removed due to the hierarchy of model term. It is also observed that the probability value of model terms C (0.3173) just barely above the significance threshold of 0.30. Therefore, model terms C can be accepted as valid model term.

As indicated by the small probability value, squared term indicates that the bicarbonate concentration (A^2), nitrate concentration (B^2) and light irradiance (C^2) individually contribute non-linearity effect in biomass productivity of *Chlorella vulgaris* when other environmental conditions remain constant. Although bicarbonate concentration, nitrate concentration and light irradiance are important environmental conditions in the cultivation, MANOVA result shows that light irradiance is the only environmental condition, which affects biomass productivity. Besides that, it is also found out that none of the interaction between environmental conditions affects biomass productivity of *Chlorella vulgaris*.

MANOVA result highlights that light irradiance is important environmental condition in regulating biomass productivity of *Chlorella vulgaris*. Besides that, combination of environmental conditions shows no significant contribution in biomass productivity of *Chlorella vulgaris*.

4.4.3 Lipid Productivity of Chlorella Vulgaris

The mathematical model (Equation 4-3) was derived for lipid productivity fitted in terms of actual factors was as follow:

$$Lipid\ productivity = -2.57549 + 0.64571A + 2.64306B + 5.56758x10^{-4}C - 0.0668AB + 2.648x10^{-5}AC - 0.0644A^2 - 1.17159B^2 - 7.577x10^{-8}C^2 \quad (4\text{-}3)$$

Figure 4-24 and Figure 4-25 display the normal plot of residuals and scatter plot of residuals versus predicted of lipid productivity (Central composite design) respectively. As shown in Figure 4-24, the residuals line up on the normal plot while residuals distributed evenly in Figure 4-25. Therefore, response transformation is not required for this experimental result of lipid productivity.

The MANOVA result is shown in the Table 4-3 where A, B and C represent bicarbonate concentration, nitrate concentration and light irradiance respectively. When the mathematical model for lipid productivity is developed with model terms of A, B, C, AB, AC, BC, A^2, B^2, C^2 and ABC, it is found out that the model fitness is very poor. Hence, model terms of AC and ABC with high probability value are omitted from lipid productivity mathematical model and MANOVA. Although model terms of B and C presents high probability value in MANOVA, both model terms of B and C should be maintained in the mathematical model without affecting model structure.

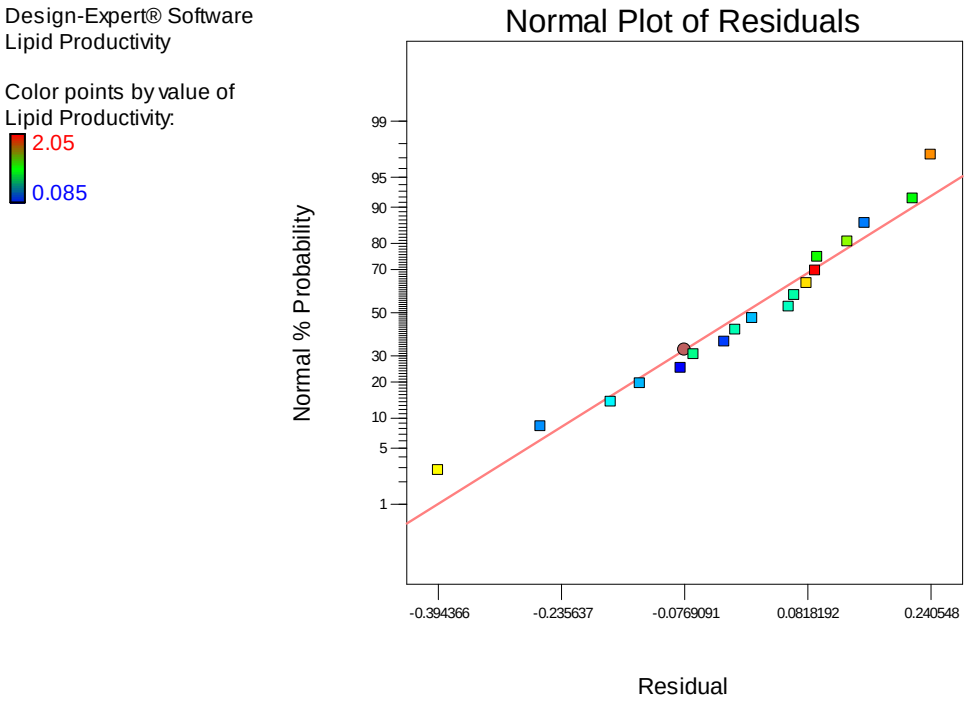

Figure 4-24: Normal plot of residuals of lipid productivity (Central composite design)

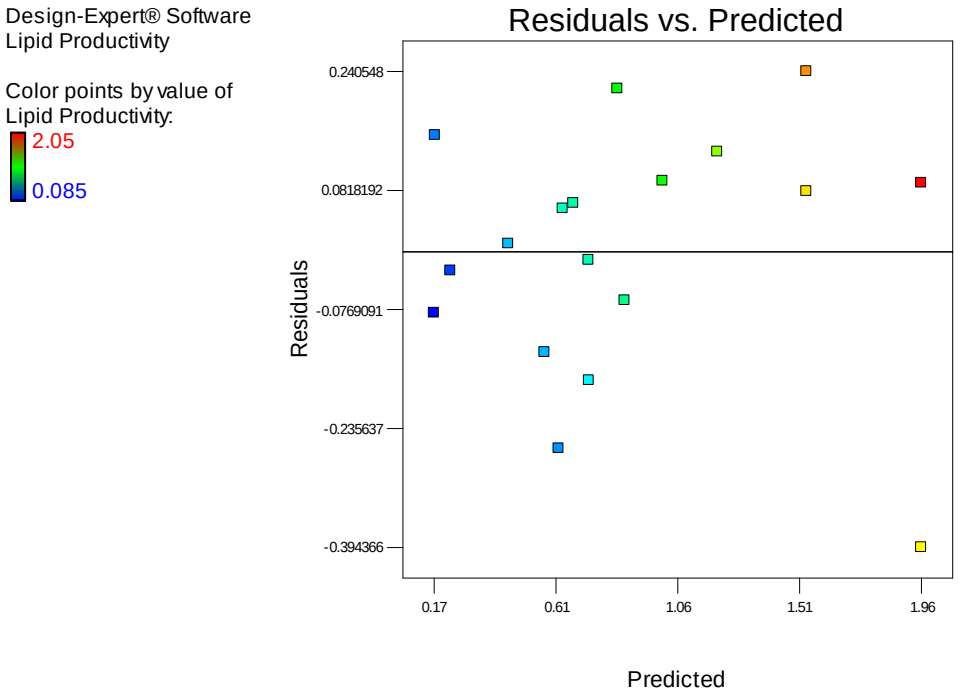

Figure 4-25: Scatter plot of residuals versus predicted value of lipid productivity (Central composite design)

Table 4-3: Multivariate analysis of variance for lipid productivity (Central composite design)

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	Significance
Block	0.4400	1	0.4400			
Model	4.8100	8	0.6000	10.40	0.0017	significant
A-Bicarbonate	0.2500	1	0.2500	4.32	0.0712	
B-Nitrate	0.0040	1	0.0040	0.07	0.7997	
C-Light Irradiance	0.0045	1	0.0045	0.08	0.7882	
AB	0.0560	1	0.0560	0.97	0.3545	
AC	0.2200	1	0.2200	3.79	0.0873	
A^2	2.0400	1	2.0400	35.27	0.0003	
B^2	1.0800	1	1.0800	18.68	0.0025	
C^2	2.8200	1	2.8200	48.82	0.0001	
Residual	0.4600	8	0.0580			
Lack of Fit	0.3300	6	0.0550	0.84	0.6319	not significant
Pure Error	0.1300	2	0.0650			
Cor Total	5.7000	17				
R-Squared	91.23%					

In Table 4-3, significant threshold of 0.30 is set. Model probability value of 0.0017 (Table 4-3) indicates that the model is significant and non-significance of Lack of Fit is supported by the probability value of 0.6319. The R-squared of 91.23% implies that the model fits the data well.

It is observed that in Table 4-3 that the probability value of AB (0.3545) is above the significance threshold of 0.30 and model term AB can easily pass the significance test. Therefore, the valid model terms are A (probability value of 0.0712), AB, AC (probability value 0.0873), A^2 (probability value of 0.0003), B^2 (probability value of 0.0025) and C^2 (probability value of 0.0001).

Squared terms of bicarbonate concentration, nitrate concentration and light irradiance implies that the change of each environmental condition produces quadratic effect in lipid productivity when other environmental conditions remain constant. Based on the smaller probability value (0.0712), bicarbonate concentration shows the great importance in the lipid productivity of *Chlorella vulgaris* during cultivation. Besides

that, the combination of bicarbonate concentration and light irradiance demonstrates strong interaction in the lipid productivity of *Chlorella vulgaris*, as supported by the probability value of 0.0873. On the other hand, the combination of bicarbonate concentration and nitrate concentration produces the least contribution in lipid productivity of *Chlorella vulgaris*, as remarked by the probability values (0.3545). These observations were supported by the findings in section 4.3.3.

It is concluded that MANOVA result demonstrated that bicarbonate concentration is important environmental conditions in affecting lipid productivity of *Chlorella vulgaris*. Besides that, the combinations of environmental conditions, which are (a) bicarbonate concentration and nitrate concentration, and (b) combination of bicarbonate and light irradiance, have strong effect in enhancing lipid accumulation of *Chlorella vulgaris*, even though nitrate concentration and light irradiance are not the individual environmental condition in affecting lipid productivity of *Chlorella vulgaris*.

4.5 Model Validation

Under effects of bicarbonate concentration, nitrate concentration and light irradiance, three different mathematical models of growth rate, biomass productivity and lipid productivity were generated from central composite design using MANOVA in section 4.4. In order to validate the mathematical model, a total of 18 experiments with random experimental configuration were performed within designated range of three environmental conditions. The obtained experimental result from random experimental configuration was used to validate the predicted response generated from mathematical model by using statistical method.

4.5.1 Validation on Growth Rate Model of Chlorella Vulgaris

The validation data was compared with predicted value from the generated model (Equation 4-1), as displayed in the Table 4-4. It is observed in Table 4-4 that the range of validation data of growth rate (0.289 day^{-1} to 0.815 day^{-1}) was not significantly different compared to the range of predicted value (0.221 day^{-1} to 0.728 day^{-1}). Since

this study involving biological activities have problems ensuring consistency and accuracy in results obtained, it is acceptable that the difference between predicted value and experimental data varied between 3 % and 20 %.

Figure 4-26 shows that the normal plot of residual which were between validation data and predicted value from generated model. It is observed in Figure 4-26 that the residuals were closed to straight line of normality. On the other hand, Figure 4-27 shows that the scatter plot of residuals versus predicted value. The residuals were evenly distributed, as observed in Figure 4-27. These observations show that the residuals behave normality and the variance of residual was not constant. Hence, the model was successfully validated.

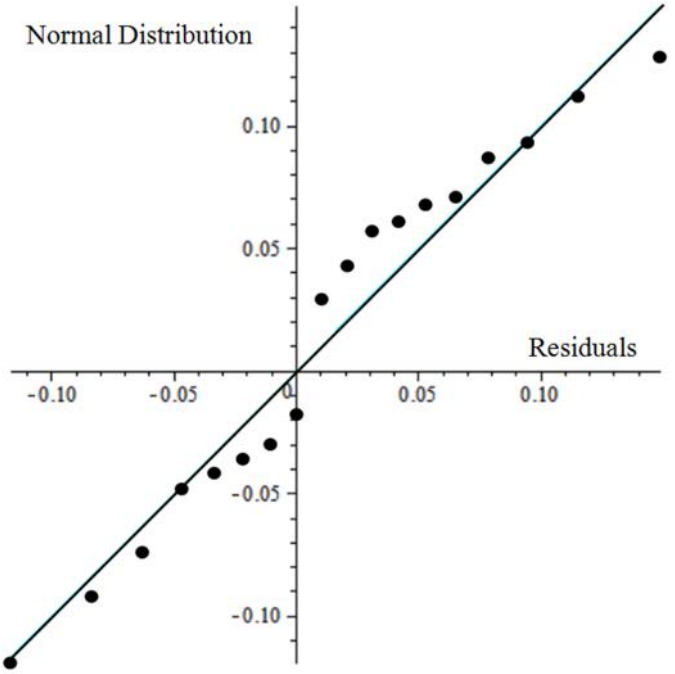

Figure 4-26: Normal plot of residuals of growth rate (Model validation)

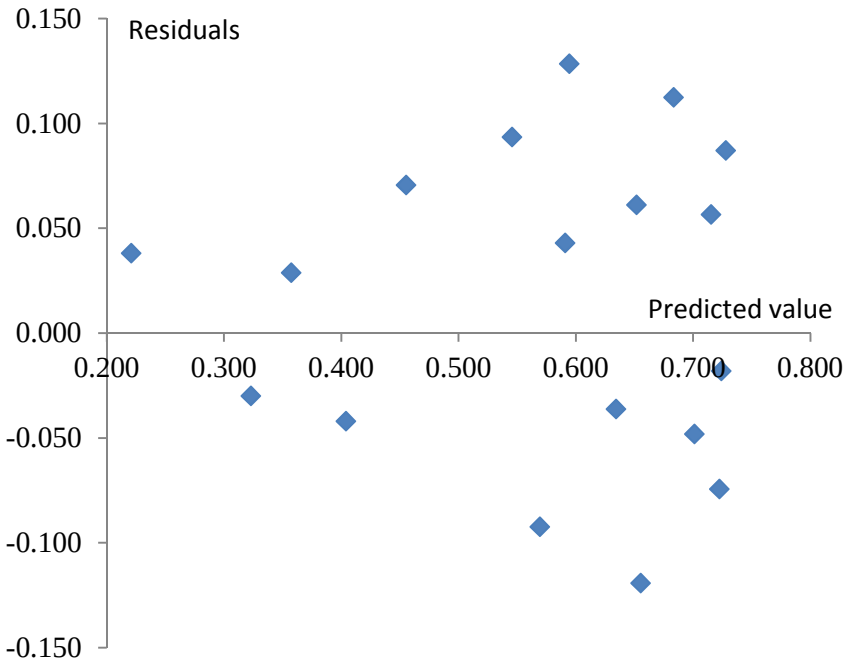

Figure 4-27: Scatter plot of residuals versus predicted value of growth rate (Model validation)

Table 4-4: Comparison between validation data and predicted value of growth rate (Central composite design model)

Factor 1 A:Bicarbonate g/L	Factor 2 B:Nitrate g/L	Factor 3 C:Light irradiance LUX	Validation data Growth rate (day-1)	Predicted value Growth rate (day-1)	Residual	Difference between validation data and predicted value (%)
6.9	0.5	2000	0.293	0.323	-0.030	10
6.9	0.5	7000	0.259	0.221	0.038	16
6.9	1.5	2000	0.386	0.357	0.029	8
5.1	1.0	4400	0.815	0.728	0.087	11
4.9	1.0	4700	0.706	0.724	-0.018	3
5.5	1.0	4600	0.648	0.722	-0.074	11
5.8	1.0	4100	0.772	0.715	0.057	8
4.4	1.3	5600	0.598	0.634	-0.036	6
3.9	1.0	4700	0.653	0.701	-0.048	7
6.3	0.7	3300	0.723	0.595	0.128	19
2.5	0.6	2700	0.526	0.455	0.071	14
5.5	1.0	6600	0.477	0.569	-0.092	18
5.9	0.9	5100	0.796	0.684	0.112	15
6.1	1.5	2100	0.362	0.404	-0.042	11
3.2	1.1	5900	0.634	0.591	0.043	7
4.4	0.8	3200	0.536	0.655	-0.119	20
3.8	1.3	6300	0.639	0.546	0.093	16
4.6	0.7	3700	0.713	0.652	0.061	9

4.5.2 Validation on Biomass Productivity Model of Chlorella Vulgaris

Comparison was made between validation data and predicted value of the biomass productivity (Equation 4-2), as shown in Table 4-5. The range of validation data (112.3 mg/L·day to 315.8 mg/L·day) was not significantly different with the range of predicted value (119.5 mg/L·day to 289.2 mg/L·day). In this study, it is very hard to obtain accurate and constant biological result. Hence, it is acceptable that the difference between predicted values and validation data was ranged from 3% to 15%. in Table 4-5

Figure 4-28 shows that the normal plot of residuals which were between validation data and predicted value. On the other hand, Figure 4-29 displays the scatter plot of residual versus predicted value. Residuals were normally distributed in the Figure 4-28 while the residuals were evenly distributed in Figure 4-29. These observations showed that the model was successfully validated by validation data.

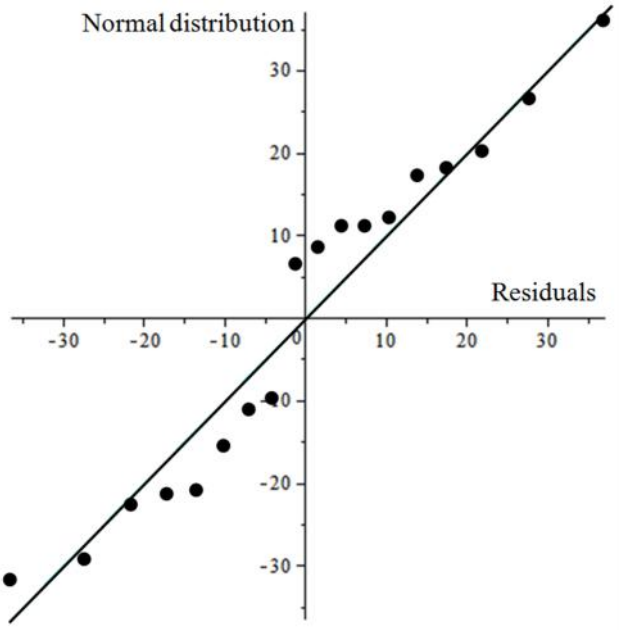

Figure 4-28: Normal plot of residuals of biomass productivity (Model validation)

Table 4-5: Comparison of biomass productivity between validation data and predicted value from central composite design model

Factor 1	Factor 2	Factor 3	Validation data	Predicted value		
A:Bicarbonate	B:Nitrate	C:Light irradiance	Biomass productivity	Biomass productivity	Residual	Difference between validation data and predicted value
g/L	g/L	LUX	(mg/L· day)	(mg/L· day)		(%)
6.9	0.5	2000	130.6	119.5	11.1	9
6.9	0.5	7000	112.3	127.8	-15.5	13
6.9	1.5	2000	143.2	123.1	20.1	15
5.1	1.0	4400	296.5	287.9	8.6	3
4.9	1.0	4700	315.8	289.2	26.6	9
5.5	1.0	4600	276.3	286.0	-9.7	3
5.8	1.0	4100	289.3	278.2	11.1	4
4.4	1.3	5600	278.4	261.2	17.2	6
3.9	1.0	4700	258.4	281.0	-22.6	8
6.3	0.7	3300	250.1	232.0	18.1	7
2.5	0.6	2700	173.3	166.7	6.6	4
5.5	1.0	6600	214.7	236.0	-21.3	9
5.9	0.9	5100	264.3	275.4	-11.1	4
6.1	1.5	2100	127.4	148.2	-20.8	15
3.2	1.1	5900	280.4	244.4	36.0	14
4.4	0.8	3200	264.5	252.4	12.1	5
3.8	1.3	6300	198.4	230.1	-31.7	15
4.6	0.7	3700	232.4	261.6	-29.2	12

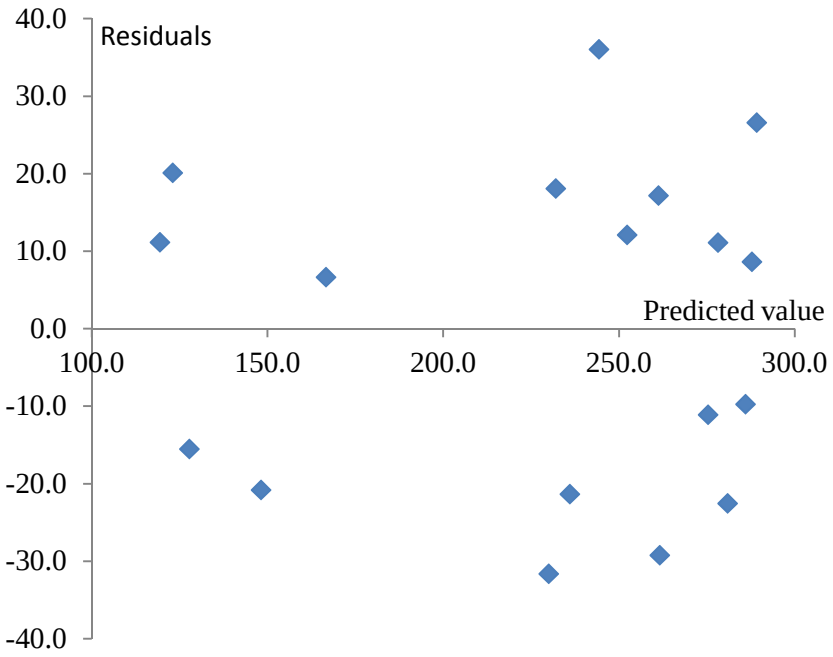

Figure 4-29: The scatter plot of residuals versus predicted value of biomass productivity (Model validation)

4.5.3 Validation on Lipid Productivity Model of Chlorella Vulgaris

Table 4-6 displays the comparison of lipid productivity between validation data and predicted value of central composite design. It is observed in Table 4-6 that validation data of lipid productivity, varied from 0.613 mg/L·day to 1.834 mg/L·day, was not significantly different from the predicted value (0.627 mg/L·day to 1.761 mg/L·day). In this study, the biological experiment has difficulty in ensuring consistency in results obtained. Hence, it is acceptable that the difference between validation data and predicted value from the mathematical model ranged from 1 % to 25 %.

Figure 4-30 displays the normal plot of residuals which was between predicted value and validation data. On the other hand, Figure 4-31 shows the scatter plot of residuals versus predicted value of lipid productivity. Figure 4-30 showed that the residuals, which were closed to straight line of normality, were normally distributed. On the other hand, Figure 4-31 showed that the residuals, were evenly distributed in the plot. This demonstrated

that the variance of residuals was not constant. Hence, as supported by these observations, the mathematical model was successfully validated.

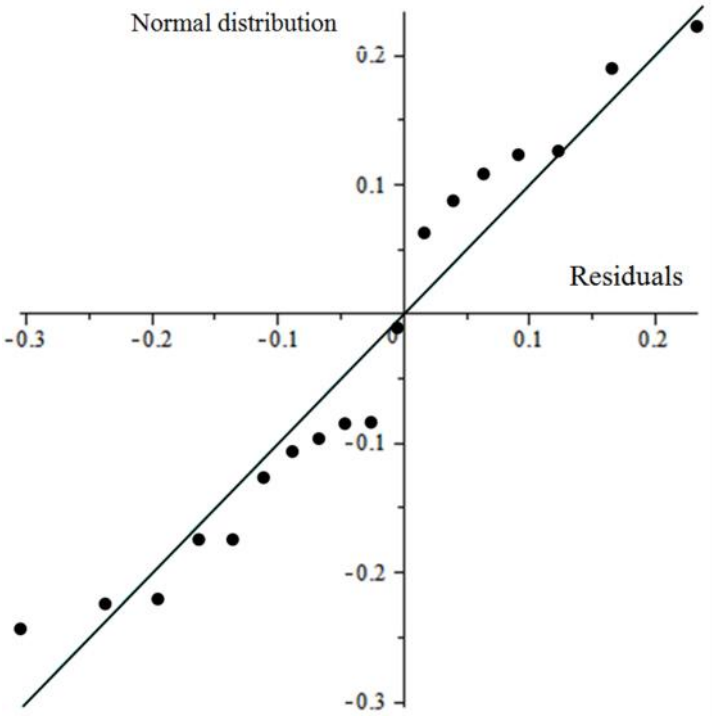

Figure 4-30: Normal plot of residuals of lipid productivity (Model validation)

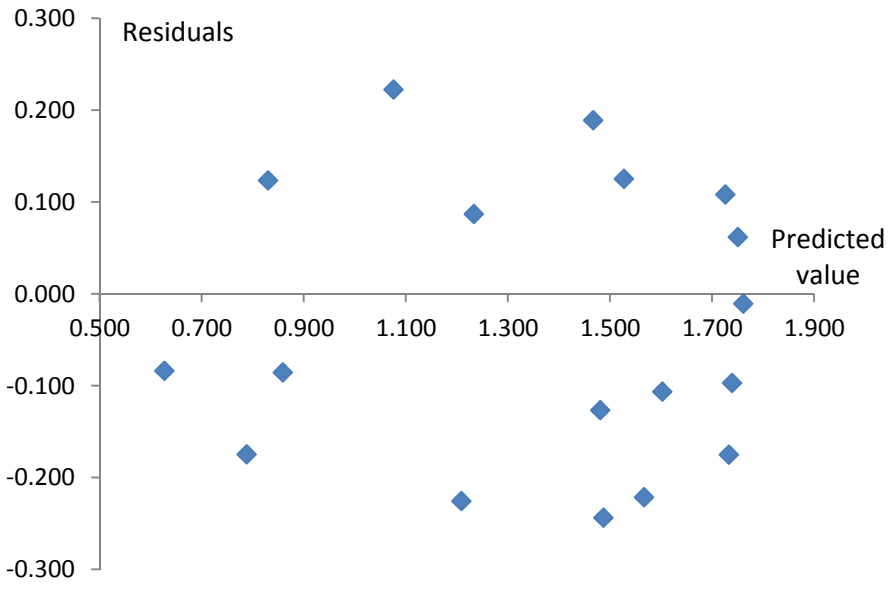

Figure 4-31: Scatter plot of residuals versus predicted value of lipid productivity (Model validation)

Table 4-6: Comparison of lipid productivity between validation data and predicted value (Central composite design model)

Factor 1 A:Bicarbonate g/L	Factor 2 B:Nitrate g/L	Factor 3 C:Light irradiance LUX	Validation data Lipid productivity (mg/L· day)	Predicted value Lipid productivity (mg/L· day)	Residual	Difference between validation data and predicted value (%)
6.9	0.5	2000	0.613	0.788	-0.175	25
6.9	0.5	7000	1.298	1.076	0.222	19
6.9	1.5	2000	0.543	0.627	-0.084	14
5.1	1.0	4400	1.812	1.750	0.062	3
4.9	1.0	4700	1.642	1.739	-0.097	6
5.5	1.0	4600	1.750	1.761	-0.011	1
5.8	1.0	4100	1.834	1.726	0.108	6
4.4	1.3	5600	1.243	1.487	-0.244	18
3.9	1.0	4700	1.496	1.603	-0.107	7
6.3	0.7	3300	1.354	1.481	-0.127	9
2.5	0.6	2700	0.953	0.830	0.123	14
5.5	1.0	6600	1.656	1.467	0.189	12
5.9	0.9	5100	1.558	1.733	-0.175	11
6.1	1.5	2100	0.773	0.859	-0.086	10
3.2	1.1	5900	1.320	1.233	0.087	7
4.4	0.8	3200	1.652	1.527	0.125	8
3.8	1.3	6300	0.983	1.208	-0.225	21
4.6	0.7	3700	1.345	1.566	-0.221	15

4.6 Discussion on Interactions between Environmental Conditions

Three mathematical models were developed to illustrate *Chlorella vulgaris* growth under effects of bicarbonate concentration, nitrate concentration and light irradiance. With 18 sets of experimental result with random experimental configurations, the fitness of estimated model was successfully validated with predicted responses generated from the model. The important interactions between environmental conditions, which were identified in MANOVA, were (i) interaction between nitrate concentration and light irradiance on growth rate of *Chlorella vulgaris*, (ii) interaction between bicarbonate and nitrate concentrations on growth rate of *Chlorella vulgaris*, (iii) interaction between bicarbonate and nitrate concentrations on lipid productivity of *Chlorella vulgaris*, and (iv) interaction between bicarbonate concentration and light irradiance on lipid productivity of *Chlorella vulgaris*. Interactions between environmental conditions were discussed in this section.

4.6.1 Interactions between Environmental Conditions on Growth Rate of Chlorella Vulgaris

Contour graph can clearly display the change of predicted value of generated model by using contour lines, which represent the different level of factor. Figure 4-32 displays the contour graph of growth rate between nitrate concentration and light irradiance. Contour graph exhibits gradient ascent toward centre and the highest growth rate was focused at the middle of contour graph, as shown in Figure 4-32. The optimum nitrate concentration and light irradiance are approximately 1 g/L and 4500 LUX respectively.

In microalgae, large quantity of nitrogen is required because nitrogen is an important constituent of amino acid, building block of biomass, chlorophyll, chloroplast and RuBisCo [31,69,71]. In order to assimilate nitrogen element, one molecule of nitrate in culture medium was reduced to one molecule of ammonium by nitrate/nitrite reductase

using six molecules of reduced ferredoxin and one molecule of nicotinamide adenine dinucleotide phosphate (NADPH). NADPH can be obtained from light dependent process. The chlorophyll in chloroplast absorbed light irradiance of 4500 LUX and stored energy in the molecules of nicotinamide adenine dinucleotide phosphate (NADPH) and adenosine triphosphate (ATP). Then, ammonium was assimilated via glutamine oxoglutarate aminotransferase (GOGAT) pathway under glutamine synthetase catalyst to produce glutamine which is made up of carbon and nitrogen source for the biosynthesis of most amino acid. With the production of amino acid, more photosynthesis apparatus and lipid will be produced within the cell in order to fulfil requirement for the mitosis of *Chlorella vulgaris*. In order to enhance the growth rate of *Chlorella vulgaris*, nitrogen source and light irradiance should be increased simultaneously. As shown in the Figure 4-32, interaction between nitrate concentration and light irradiance on growth rate of *Chlorella vulgaris* become stronger when nitrate concentration and light irradiance were increased to 1 g/L and 4500 LUX respectively.

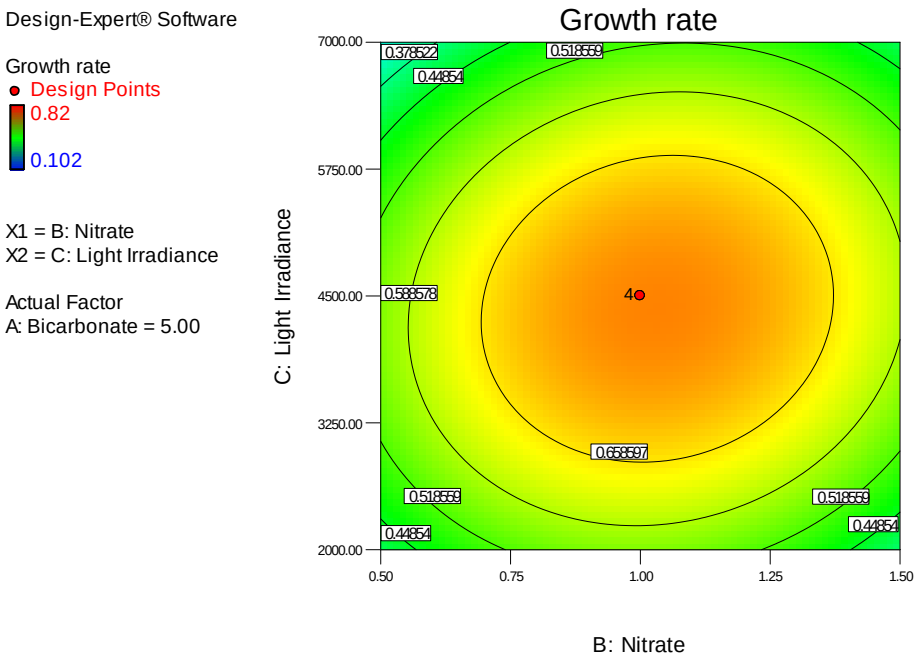

Figure 4-32: Contour graph for growth rate with the combination of nitrate concentration and light irradiance

Another contour graph of growth rate between bicarbonate and nitrate concentrations is shown in Figure 4-33. Same observation was found in the Figure 4-33 whereby the contour graph demonstrates gradient ascent toward the centre and the highest growth rate also centred in the contour graph. The optimum values for bicarbonate and nitrate concentrations are estimated at 5 g/L and 1 g/L respectively.

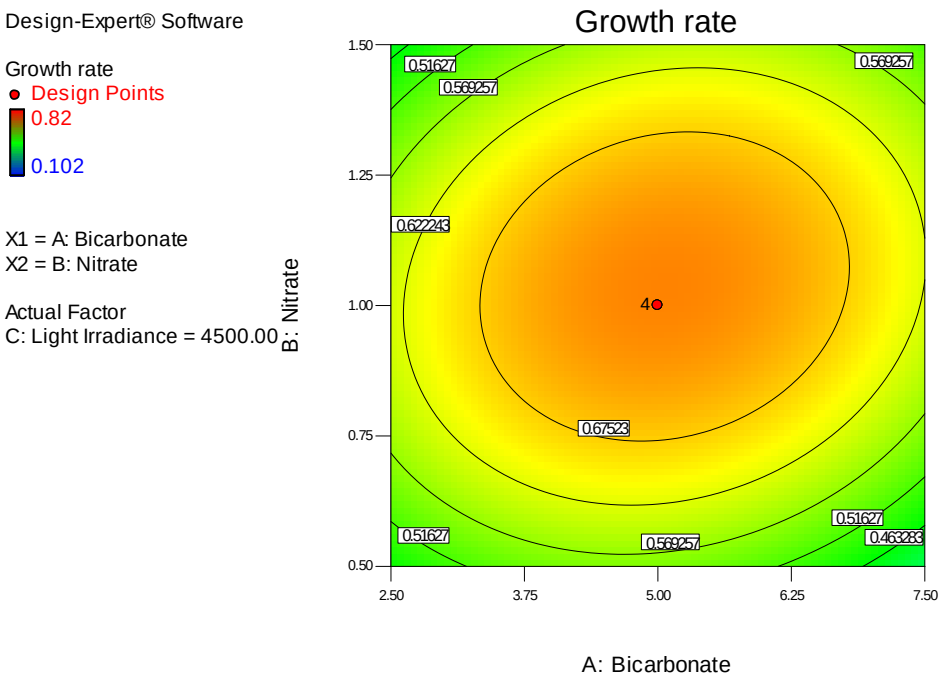

Figure 4-33: Contour graph of growth rate with the combination of bicarbonate and nitrate concentrations

Growth and development of *Chlorella vulgaris* were highly dependent on the interaction between carbon and nitrogen metabolism. Large amounts of nitrogen are invested in photosynthetic machinery which are RuBisCo and light harvesting complex. The photosynthetic machinery assimilated carbon dioxide aqueous and produced fixed carbon from Calvin's cycle. Carbon skeletons produced by fixed carbon assimilated nitrogen from nitrate to produce amino acid. Production of amino acid plays crucial role as building block of photosynthesis machinery which are protein, enzyme, chloroplast,

chlorophyll and nucleic acid. As shown in Figure 4-33, the growth rate of *Chlorella vulgaris* was improved when bicarbonate concentration and nitrate concentration were increased to 5 g/L and 1 g/L respectively. This showed that interaction between carbon and nitrogen metabolism contributed stronger interactive effect in growth rate of *Chlorella vulgaris*.

However, extremely low or excess quantity of environmental condition could weaken both interactions on growth rate of *Chlorella vulgaris*, as shown in Figure 4-32 and Figure 4-33. With low nitrate concentration, nitrate and nitrite reductase can only generate limited concentration of ammonium [35] for glutamine oxoglutarate aminotransferase (GOGAT) pathway. Low production of glutamine from GOGAT pathway led to low production of protein, enzyme, chlorophyll, chloroplast, and nucleic acid. This greatly inhibited *Chlorella vulgaris* growth although the supply of other environmental condition is sufficient.

When excess nitrate concentration (higher than 1 g/L) is applied in the beginning of the cultivation, the rate of ammonium production from nitrate/nitrite reductase was more than nitrogen assimilation with low cell population [17,49]. Excess ammonium accumulated in cell could trigger uncoupling of phosphorylation resulting with adenosine triphosphate (ATP) deficiency. ATP deficiency could inhibit the operation of GOGAT pathway, nitrogen assimilation and Calvin's cycle. Hence, growth rate was gradually decreased when nitrate concentration was decreased from 1.0 g/L to 0.5 g/L and was increased from 1 g/L to 1.5 g/L, as shown in Figure 4-32 and Figure 4-33.

Weak light irradiance can be absorbed by chlorophyll but longer time is needed by chloroplast to absorb weak light irradiance for the synthesis of adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide phosphate (NADPH) [66]. In this study, at the beginning of cultivation, it was observed that light irradiance of 2000 LUX can penetrate 10 cm width of flat-panel photo-bioreactor from one glass wall to another one. This allowed that chloroplast of low cell population had sufficient time absorbing weak

light irradiance for light dependent process. However, as the cultivation was continuously carried out few days, cell population was increasing and the penetration ability of weak light irradiance was weakened. More time is required by chloroplast to absorb weak light irradiance to produce ATP and NADPH supporting Calvin's cycle. Hence, the growth of Chlorella vulgaris was gradually inhibited at low light irradiance, as shown in Figure 4-32. Strong light irradiance (higher than 4500 LUX) could damage the chlorophyll, photosynthesis apparatus. Consequently, Calvin's cycle and nitrogen assimilation cannot be fuel because the conversion of light energy into chemical energy was inhibited by strong light irradiance. However, the growth rate of *Chlorella vulgaris* was improved when light irradiance was increased from 2000 LUX to 4500 LUX.

Limited carbon source was obtained from low bicarbonate concentration for carbon fixation process. The production of organic compound from carbon fixation process was gradually inhibited as the concentration of carbon source in medium was decreasing. In order to enhance growth rate of *Chlorella vulgaris*, increase in bicarbonate concentration from 2.5 g/L to 5 g/L could enhance growth rate of *Chlorella vulgaris*, as shown in Figure 4-33. On the other hand, it is reported that *Chlorella* can withstand high carbon dioxide concentration up to 70% (v/v%) [89,90,106]. High bicarbonate concentration was then applied to produce high concentration of carbon dioxide aqueous in the culture medium. During cultivation, the concentration of produced oxygen from photosynthesis in medium was dramatically increased due to increasing cell population. As the oxygen concentration was increased in the medium, *Chlorella* could have difficulty in exporting oxygen from cell to the medium [86]. Hence, increase in bicarbonate concentration from 5 g/L to 7.5 g/L could inhibit the growth rate of *Chlorella vulgaris* due to photorespiration.

Hence, it is demonstrated in Figure 4-32 that nitrate concentration of 1 g/L and light irradiance of 4500 LUX have strong interaction at the centre of contour graph. Besides that, bicarbonate concentration of 5 g/L and nitrate concentration of 1 g/L produce strong interaction at the centre of contour graph, as shown in Figure 4-33. However,

excess or extremely low quantity of environmental conditions should be avoided to prevent deterioration in growth rate of *Chlorella vulgaris*.

4.6.2 Interaction between Environmental Conditions on Lipid Productivity of Chlorella Vulgaris

As shown in Figure 4-34, the lipid productivity is illustrated by the interaction between bicarbonate and nitrate concentrations in contour graph. While, Figure 4-35 shows the lipid productivity described by the interaction between bicarbonate concentration and light irradiance in contour graph. The converging of contour lines towards the centre of the plot indicates that contour graphs demonstrate gradient ascent and therefore, the contour graphs have bowl shape with maximum lipid productivity at the centre of the contour graph. Figure 4-34 and Figure 4-35 show that the increase of both environmental conditions improves the lipid productivity of *Chlorella vulgaris* until maximum is reached.

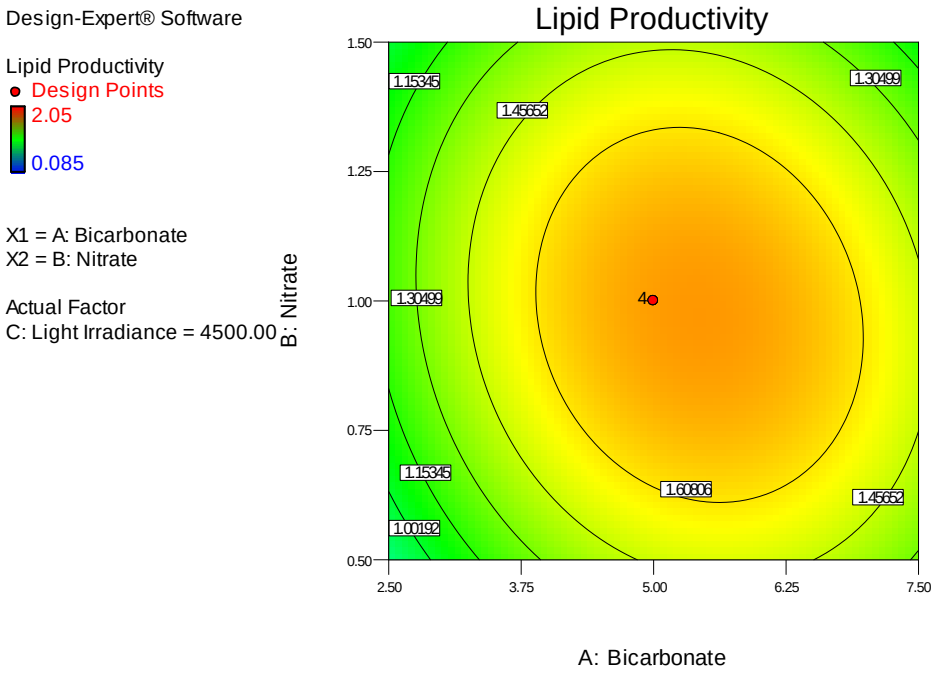

Figure 4-34: Contour graph for lipid productivity between bicarbonate and nitrate concentrations

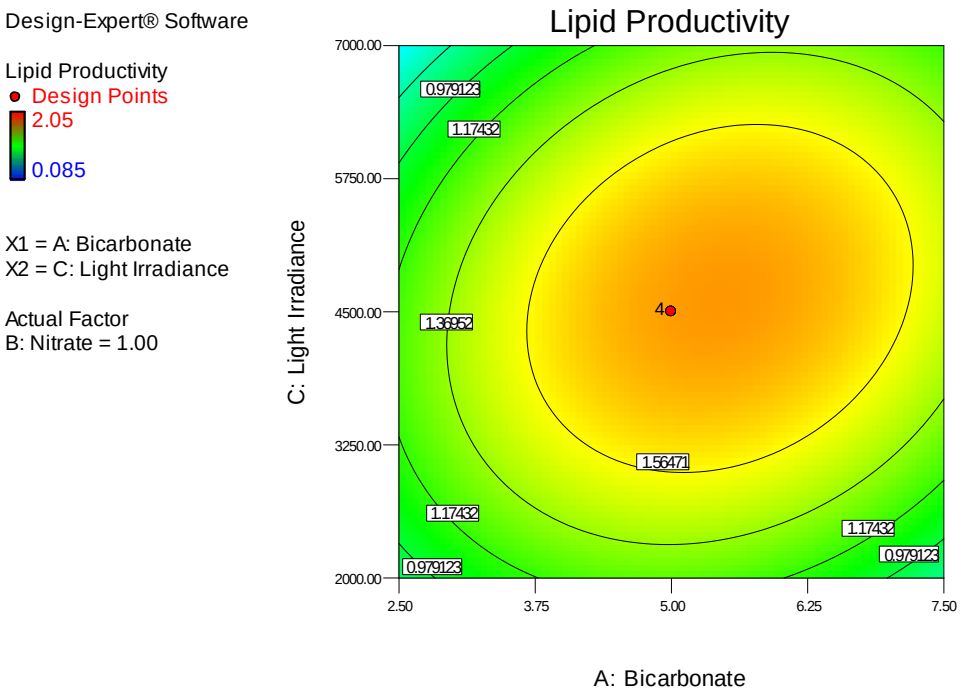

Figure 4-35: Contour graph for lipid productivity between bicarbonate concentration and light irradiance

As discussed early in section 4.6.1, it was identified that strong interaction between bicarbonate and nitrate concentrations was contributed in growth rate of *Chlorella vulgaris*. Besides that, as shown in Figure 4-34, interaction between bicarbonate and nitrate concentrations also contributed strong interactive effect on lipid production rate of *Chlorella vulgaris*. Lipid plays two important roles in *Chlorella vulgaris* as energy storage molecule and in the formation of biological membrane. In order to produce lipid, high demand for carbon source was required in carbon fixation process to produce glyceraldehyde 3-phosphate (G3P). Besides that, various catalysts supporting lipid synthesis pathway were mostly made up of amino acid which consisted of carbon and nitrogen element.

As mentioned in 4.6.1, nitrate was assimilated via nitrate/nitrite reductase to produce ammonia. After that, ammonia was assimilated under catalyst of glutamine synthetase via glutamine oxoglutarate aminotransferase (GOGAT) pathway to produce glutamate as

building block for amino acid. As shown in Figure 4-34, lipid synthesis pathway was enhanced by increasing carbon and nitrogen sources as carbon and nitrogen sources were required for carbon fixation process and GOGAT pathway respectively. This showed that interaction between bicarbonate and nitrate concentration grew stronger when bicarbonate and nitrate concentrations were increased to 5 g/L and 1 g/L respectively.

On the other hand, Figure 4-35 shows that lipid productivity was improved when bicarbonate concentration (from 2.5 g/L to 5 g/L) and light irradiance (from 2000 LUX to 4500 LUX) were increased. Lipid synthesis pathway was highly dependent on carbon source and light irradiance. Light irradiance was absorbed by chlorophyll and was stored in ATP. High demand of adenosine triphosphate (ATP) was required to fuel lipid synthesis pathway and carbon fixation process. Without ATP, carbon fixation process and lipid synthesis pathway could be inhibited. This shows that light irradiance and bicarbonate concentration have strong interactions affecting the lipid productivity of *Chlorella vulgaris* at centre of Figure 4-35.

However, as shown in Figure 4-34 and Figure 4-35, excess or extremely low bicarbonate concentration, nitrate concentration or light irradiance could upset both interactions on lipid production rate of *Chlorella vulgaris*. These observations were similar with the discussion on interaction between bicarbonate and nitrate concentration on growth rate of *Chlorella vulgaris* and, interaction between bicarbonate concentration and light irradiance on growth rate of *Chlorella vulgaris*, as discussed in section 4.6.1.

Low carbon source could lead to low production of G3P from carbon fixation process and lipid synthesis pathway could be inhibited. On the other hand, *Chlorella vulgaris* can tolerate with high concentration of carbon dioxide up to 70% (v/v%) and high bicarbonate concentration could result with high photosynthesis efficiency of *Chlorella vulgaris*. However, as the cultivation was continuously performed, oxygen concentration

in medium was increased and cell could have difficulty to export oxygen to the medium [86].

Strong light irradiance could damage the photosynthetic machinery and the conversion of light energy to chemical energy was inhibited due to photo-inhibition. On the other hand, longer exposure time is needed by chlorophyll to absorb weak light irradiance in light dependant process. At the beginning of cultivation, low light irradiance of 2000 LUX was available in photo-bioreactor with low cell density. After the cultivation was continuously carried out for few days, the increase in cell density reduced the penetration ability of light into the culture medium. Insufficient light energy was available for high cell population to produce chemical energy for lipid synthesis pathway.

On the other hand, high nitrate concentration could lead to ammonia accumulation in cell when the production rate of ammonia was higher than nitrate assimilation [17,49]. Excess ammonia accumulation could trigger uncoupling of phosphorylation resulting with ATP deficiency. Although ATP deficiency could inhibit nitrate assimilation to reduce ammonia accumulation in cell, ATP deficiency also could lead to inhibition of lipid synthesis pathway and carbon fixation process. Besides that, limited nitrogen element assimilated from low nitrate concentration could inhibit the production of glutamate as building block of amino acid via GOGAT pathway. As the production of catalyst supporting lipid synthesis pathway was inhibited, lipid synthesis pathway, carbon and nitrogen metabolism was inhibited.

It is deducted in Figure 4-34 that bicarbonate concentration of 5 g/L and nitrate concentration of 1 g/L had strong interaction in lipid production of *Chlorella vulgaris*. It is also demonstrated in Figure 4-35 that bicarbonate concentration of 5 g/L and nitrate concentration of 1 g/L produced strong interaction at the centre of the contour graph. However, lipid production could be inhibited by excess or extremely low quantity of environmental conditions.

4.7 Optimization

The optimum setting of environmental conditions can be generated through the objective function. In this study, the lipid productivity of *Chlorella vulgaris* becomes the optimization target, which needs to be maximized, in order to meet the research objective.

The 3D surface curve plots were plotted to explain the significant interactions of environmental conditions and the optimum setting of each parameter required for the purpose of maximising lipid productivity of *Chlorella vuglaris*. Figure 4-36 shows the 3D surface curve plot for lipid productivity between bicarbonate and nitrate concentrations. On the other hand, 3D surface curve plot for lipid productivity between bicarbonate concentration and light irradiance is shown in Figure 4-37.

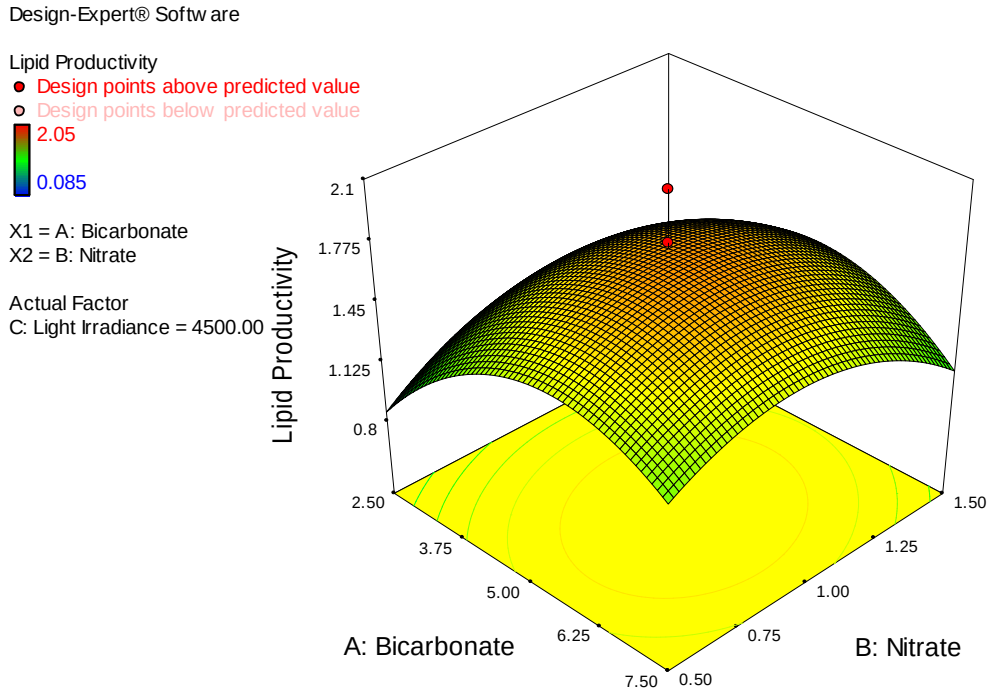

Figure 4-36: 3D surface curve plot for lipid productivity between bicarbonate and nitrate concentrations at light irradiance of 4500 LUX.

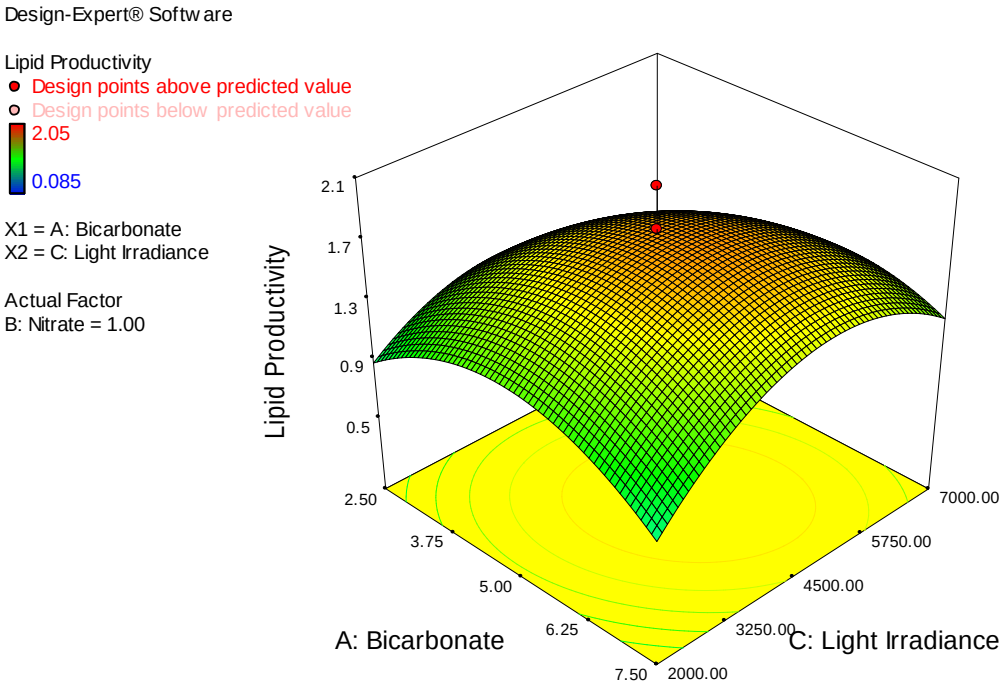

Figure 4-37: 3D surface curve plot for lipid productivity between bicarbonate concentration and light irradiance at 1 g/L nitrate concentration.

It is observed in Figure 4-36 and Figure 4-37 that the surface of the both the 3D surface curve plots inclines toward the centre of the plot and therefore, both 3D surface curve plot have bowl shape with maximum lipid productivity of *Chlorella vulgaris* shown at the centre of both 3D surface curve plots. Figure 4-36 and Figure 4-37 shows that the increase of both environmental conditions improves the lipid productivity. However, the lipid productivity is deteriorated when both environmental conditions are further increased. Besides that, strong interaction between environmental conditions is presented at the centre of the plot in Figure 4-36 and Figure 4-37.

Similar observation is shown between Figure 4-34 and Figure 4-36, as discussed earlier in section 4.6.2. When the cell growth is improved by increasing nitrate concentration (from 0.5 g/L to 1 g/L), higher bicarbonate concentration (from 2.5 g/L to 5 g/L) is required for lipid production. However, further increase in bicarbonate (from 5 g/L to

7.5 g/L) and nitrate (from 1 g/L to 1.5 g/L) concentrations results in low lipid production due to cell growth inhibition.

On the other hand, Figure 4-37 also shows similar observation with Figure 4-35, as discussed earlier in section 4.6.2. Lipid accumulation could be enhanced at high light irradiance (from 2000 LUX to 4500 LUX) when the bicarbonate concentration (from 2.5 g/L to 5 g/L) is higher. Similar finding was shown by Yeh et al. (2010) that the carbon source utilisation is increased with the increase in light irradiance. Hence, it is also deduced that the bicarbonate concentration and light irradiance have strong interaction that affect the lipid productivity of *Chlorella vulgaris*. However, the lipid productivity is deteriorated when bicarbonate concentration (from 5 g/L to 7.5 g/L) and light irradiance (2000 LUX to 7000 LUX) are further increased.

As shown in Figure 4-34 and Figure 4-36, the highest lipid productivity was roughly pinpointed at the centre of the contour plot and 3D surface curve plot. The optimum bicarbonate concentration is located around 5 g/L and the optimum light irradiance is approximately 4500 LUX. On the other hand, as shown in Figure 4-35 and Figure 4-37, the maximum lipid productivity was estimated at the middle of the contour graph and 3D surface curve plot, which might be 5 g/L bicarbonate concentration and 1 g/L nitrate concentration. It is shown in Figure 4-34, Figure 4-35, Figure 4-36 and Figure 4-37 that the strong interaction between environmental conditions is demonstrated at the centre of the contour graph and 3D surface curve plot. Hence, it is necessary to perform optimization to obtain optimum process setting for maximum lipid productivity.

The objective function of this optimization is the mathematical model (Equation 4-3), which was generated to predict the lipid productivity of *Chlorella vulgaris*, where A, B and C represent bicarbonate concentration, nitrate concentration and light irradiance respectively. On the basis of optimization, the extrapolated range of the central composite design was neglected and the designated range of each environmental condition are 2.5 – 7.5 g/L bicarbonate concentration, 0.5 – 1.5 g/L nitrate concentration

and light irradiance of 2000 – 7000 LUX. Therefore, the optimization problem was formulated so as to maximize the lipid productivity of *Chlorella vulgaris*.

Maximise:

$$Lipid\ productivity = -2.57549 + 0.64571A + 2.64306B + 5.56758x10^{-4}C - 0.0668AB + 2.648x10^{-5}AC - 0.0644A^2 - 1.17159B^2 - 7.577x10^{-8}C^2$$

Subject to:

$2.5 \leq A \leq 7.5$

$0.5 \leq B \leq 1.5$

$2000 \leq C \leq 7000$

Simplex optimization is efficient approach to determine optimum setting for this optimization problem. In simplex optimization, 39 different starting points lead to one global extreme. Within the experimental ranges, the optimum experiment configuration (5.46 g/L bicarbonate concentration, 0.97 g/L nitrate concentration and light irradiance of 4600 LUX) and optimum response of 1.756 mg/L·day were suggested by the desirability value of 85.3%. The desirability value, which is well above the satisfactory limit of 85%, implies that these optimum environmental conditions would have higher success rate to produce maximum lipid productivity. Besides that, the optimum process setting, which was generated from simplex optimization, was supported by the observations from Figure 4-36 and Figure 4-37.

4.8 Validation on Optimized Experimental Configuration

To confirm the validity of the optimized grow parameter settings, two same experiments were performed based on the optimized experimental configuration (5.46 g/L bicarbonate concentration, 0.97 g/L nitrate concentration and light irradiance of 4600

LUX). The comparison between the collected experimental data and optimum response is displayed in Table 4-7.

Table 4-7: Comparison between experimental data and optimum response of lipid productivity

Factor 1	Factor 2	Factor 3	Experiment-al Run	Confirmation Run	Optimum Response	Difference between experiment-al data and predicted value
Bicarbo-nate	Nitrate	Light irradiance	Lipid productivity	Lipid productivity	Lipid productivity	
g/L	g/L	LUX	(mg/L·day)	(mg/L·day)	(mg/L·day)	(%)
5.46	0.97	4600	1.564	1.687	1.756	4.0
5.46	0.97	4600	2.050	1.813	1.756	3.2

From optimized experimental configuration, the obtained confirmation run data were 1.687 mg/L·day and 1.813 mg/L·day and the difference between the confirmation run data and predicted data ranged 3 % to 4 %. It is observed in Table 4-7 that the confirmation run data of lipid productivity were considered relatively close to the optimum response. However, confirmation runs notably did not produce significant improvement in lipid productivity compared to experimental data and optimum response because the maximum lipid productivity of experimental data was between 1.564 mg/L·day to 2.050 mg/L·day, as shown in Table 4-7. Since experiments involving biological activity have the difficulty in ensuring consistency in the obtained results, it was reasonable as long as the result of confirmation runs fall within the range of the maximum lipid productivity of experimental data. Besides that, the optimized experimental configuration is close to the experimental configuration (5 g/L bicarbonate concentration, 1 g/L nitrate concentration and 4500 LUX), which produces the maximum lipid productivity (Table 4-7). Therefore, the maximum lipid productivity of initial experiment runs could be the optimum itself before optimization was applied.

From the comparison of experimental configurations (optimum and maximum lipid productivity), the bicarbonate concentration and light irradiance were differed by 0.5 g/L and 127 LUX respectively. To reduce the raw material cost, it is suggested that optimum

bicarbonate concentration can be replaced by 5 g/L bicarbonate concentration. On the other hand, the difference between 4500 LUX and 4600 LUX is very minor and therefore, applying 4500 LUX also can produce high lipid productivity and reduce utility cost simultaneously. Therefore, 5 g/L bicarbonate concentration, 0.97 g/L nitrate concentration and light irradiance of 4500 LUX can be used as new optimized experimental.

4.9 Discussion on Optimization

Considering the costs, the new optimized experimental configuration for lipid productivity of *Chlorella vulgaris* was set to be 5 g/L bicarbonate concentration, 0.97 g/L nitrate concentration and light irradiance of 4500 LUX in this study. The lipid productivity of *Chlorella vulgaris* from the confirmation runs was obtained around 1.750 mg/L·day.

Two similar research studies [13,105] found out that the lipid productivity of *Chlorella vulgaris* was related to bicarbonate concentration, nitrate concentration and light irradiance in culture medium. In the literature delivered by Yeh et al. (2010), under supporting of 1.25 g/L nitrate concentration in cylindrical vessel photo-bioreactor, the lipid productivity of *Chlorella vulgaris* was 39.55 mg/L·day when the optimal bicarbonate and light irradiance were 1 g/L and 3072 LUX respectively. Besides that, another similar research, which was conducted by Chen et al. (2010), also demonstrated that higher lipid productivity of 60.5 mg/L·day was obtained under the optimal bicarbonate and nitrate concentration of 1.5 g/L and 0.65 g/L respectively in cylindrical vessel photo-bioreactor.

It is found out that the results obtained by Yeh et al. (2010) and Chen et al. (2010) was higher than the overall results of this study. The big gap of lipid productivity between this study and literatures [13,105] could be caused by different photo-bioreactor design [1].

Various photo-bioreactor designs can provide different effect to the microalgae cultivation, which directly affects the outcome of microalgae [1]. In this study, both sides of flat panel photo-bioreactor were illuminated by using fluorescent lamp, as shown in Figure 3-2. While, the cylindrical vessel photo-bioreactors, which were used in Yeh et al. (2010) and Chen et al. (2010)'s experiment, were illuminated from the side by using fluorescent lamp. This could indicate that the exposing surface of culture medium to light irradiance in cylindrical vessel photo-bioreactor was higher compared to flat panel photo-bioreactor [50]. Increase in area of illumination allows more microalgae cell to receive light energy and perform lipid accumulation. As mentioned earlier in section 4.6, with adequate amount of light irradiance, the carbon utilisation rate of microalgae will be increased in the cultivation. In this study, only one third of the flat panel tank was filled by culture medium and the exposure area of culture medium to light irradiance in flat panel photo-bioreactor is smaller than in cylindrical vessel photo-bioreactor. This would explain the lower value of lipid productivity obtained compared to literatures [13,105]. With the consideration of quantity of culture medium, constructing cylindrical vessel photo-bioreactor is harder than flat panel photo-bioreactor [58]. And, it is also not convenient to perform cleanliness and maintenance in cylindrical vessel photo-bioreactor. Hence, flat panel photo-bioreactor is more appropriate to be used in this study.

This study provided the optimized experimental configuration as the fundamental experiment setting of the future work. It appears that this optimized experiment configuration is still hiding a wealth of potential if carbon dioxide gas supply is into considerations.

4.10 Conclusion

A 3-factor-5-level central composite design was applied to investigate the growth rate, biomass productivity and lipid productivity of *Chlorella vulgaris* by varying the bicarbonate concentration, nitrate concentration and light irradiance. The average value of the maximum growth rate, biomass productivity and lipid productivity of *Chlorella*

vulgaris obtained was 0.727 day^{-1}, 289.1 mg/L·day and 1.752 mg/L·day respectively when *Chlorella vulgaris* was grown in culture medium with 5 g/L bicarbonate concentration, 1 g/L nitrate concentration and light irradiance of 4500 LUX. Three mathematical models were developed to illustrate growth rate, biomass productivity and lipid productivity of *Chlorella vulgaris* under the effects of bicarbonate concentration, nitrate concentration and light irradiance.

Multivariate analysis of variance (MANOVA) result shows that nitrate concentration and light irradiance are the individual environmental conditions that affect the growth rate of *Chlorella vulgaris*. On the other hand, biomass productivity of *Chlorella vulgaris* can only be enhanced by light irradiance, as shown in MANOVA result. In order to enhance lipid production rate of *Chlorella vulgaris*, MANOVA result determines that bicarbonate concentration was the important environmental condition affecting lipid productivity of *Chlorella vulgaris*.

It is a new finding that the growth rate of *Chlorella vulgaris* was affected by interaction between bicarbonate and nitrate concentration, as well as interaction between nitrate concentration and light irradiance, as shown in MANOVA result. Besides that, MANOVA result identified that interaction between bicarbonate and nitrate concentrations, and interaction between bicarbonate concentration and light irradiance, were able to improve lipid production rate of *Chlorella vulgaris*. However, no interactive effect between environmental conditions on biomass productivity of *Chlorella vulgaris* was identified.

With increasing nitrate concentration (from 0.5 g/L to 1 g/L), nitrogen was assimilated to produce amino acid manufacturing photosynthetic apparatus and other cellular molecules. With the help of higher light irradiance (from 2000 LUX to 4500 LUX), more energy can be absorbed by photosynthetic apparatus to fuel nitrogen assimilation, carbon fixation process and amino acid production. Also, with higher nitrate concentration (from 0.5 g/L to 1 g/L), higher amount of bicarbonate concentration (from

2.5 g/L to 5 g/L) needed to manufacture skeleton ring produced from fixed carbon for amino acid production.

Apart from manufacturing carbon skeleton ring for amino acid production, carbon source from higher bicarbonate concentration (from 2.5 g/L to 5 g/L) was used to produce glyceraldehyde 3-phosphate (G3P) as raw material in lipid synthesis pathway. With higher nitrate concentration (from 0.5 g/L to 1 g/L), high demand of nitrogen element needed to manufacture catalysts supporting carbon fixation process and lipid synthesis pathway. On the other hand, higher bicarbonate concentration (from 2.5 g/L to 5 g/L) was required in the carbon fixation process to produce G3P for lipid synthesis pathway. During lipid accumulation, stronger light irradiance (from 2000 LUX to 4500 LUX) needed to produce ATP molecules to fuel carbon fixation process and lipid synthesis pathway.

However, further increase in bicarbonate concentration (5 g/L to 7.5 g/L), nitrate concentration (from 1 g/L to 1.5 g/L) and light irradiance (from 4500 LUX to 7000 LUX) could result in unfavourable inhibition of cell growth and lipid production.

With the application of simplex optimization, the optimized experimental configuration was 5 g/L bicarbonate concentration, 0.97 g/L nitrate concentration and light irradiance of 4500 LUX, which gave optimum average lipid productivity of 1.750 mg/L·day.

Chapter 5 Conclusions and Recommendations

5.1 Introduction

This chapter delivers the conclusions from this study on the cultivation of *Chlorella vulgaris* by varying different environmental conditions (bicarbonate concentration, nitrate concentration and light irradiance) as well as optimization of the lipid productivity. Recommendations were also suggested to improve the cultivation of *Chlorella vulgaris* in the future work.

5.2 Conclusions

Nitrate concentration and light irradiance were found to affect growth rate of *Chlorella vulgaris* individually. The growth rate of *Chlorella vulgaris* was also significantly affected by interaction between multiple environmental conditions. They are (a) bicarbonate and nitrate concentrations and (b) nitrate concentration and light irradiance.

On the other hand, bicarbonate concentration was the individual environmental condition affecting lipid productivity of *Chlorella vulgaris*. Besides that, interaction was

demonstrated by the combination of multiple environmental conditions in affecting lipid productivity. They are: (a) bicarbonate and nitrate concentrations and (b) bicarbonate concentration and light irradiance.

However, biomass productivity of *Chlorella vulgaris* was only enhanced by light irradiance. Although carbon and nitrogen source are essential nutrient for biomass production, increase in light irradiance is more important environmental in enhancing carbon source utilization.

Three statistical models were developed from central composite design and were successfully validated by experimental runs with random experimental configuration. In model validation, one local maxima was found in each developed model within designated range of environmental conditions. Growth rate, biomass productivity and lipid productivity of *Chlorella vulgaris* can be illustrated under effect of bicarbonate concentration, nitrate concentration and light irradiance.

With obtained mathematical model, lipid productivity of *Chlorella vulgaris* was optimized to improve the outcome of cultivation of *Chlorella vulgaris*. 5 g/L bicarbonate concentration, 0.97 g/L nitrate concentration and light irradiance of 4500 LUX were the optimized experimental configuration and the average lipid productivity of 1.750 mg/L·day was obtained.

5.3 Recommendations

In this study, the growth behaviour of *Chlorella vulgaris* was studied by varying bicarbonate concentration, nitrate concentration and light irradiance. Lipid productivity of *Chlorella vulgaris* was optimized and optimized experiment configuration was 5 g/L bicarbonate concentration, 0.97 g/L nitrate concentration and light irradiance of 4500 LUX. In spite of the fact that obtained lipid productivity from confirmation runs was lower than literatures [13,106], the optimized experiment configuration is served as the

fundamental experiment configuration for the further improvement and future development of the scale-up experiment.

Normally, the cultivation of microalgae was performed with the supply of carbon dioxide gas but the carbon dioxide aqueous is relatively low due to low solubility of carbon dioxide in the water. Although applying bicarbonate concentration would solve the issue of low carbon dioxide aqueous in culture medium, bicarbonate concentration would be used up with increasing cell population of *Chlorella vulgaris*. Bicarbonate concentration would provide sufficient carbon dioxide aqueous at the beginning of experiment and carbon dioxide gas can replenish the running low of carbon dioxide aqueous in the culture medium. This might increase the lipid productivity of *Chlorella vulgaris*. Therefore, it is suggested to perform the cultivation of *Chlorella vulgaris* with the supply of carbon dioxide gas together with bicarbonate powder.

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