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Optimization of Photosynthetic Hydrogen Gas Production by Green Alga in Sulfur Deprived Condition

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Abstract

Objective: To study the effects of nutrient concentrations, pH, sodium carbonate concentration and microwave irradiation on the growth and the hydrogen production rate of the algae *Chlamydomonas reinhardtii.* **Method/Analysis**: In this study the factors affecting the growth rate and hydrogen production by *C. reinhardtii* were evaluated using Response Surface Methodology (RSM). For maximum specific growth rate of the algae changes of ammonium, phosphate and sulfate concentration in the culture medium were studied. **Findings:** The optimum conditions determined were 7.2 mM of ammonium concentration, 1.81 mM of phosphate concentration and 1.89 mM of sulfate concentration with a maximum specific growth rate of 0.01662/h. Likewise, for hydrogen production rate, changes of ammonium and phosphate concentration as well as pH of the medium were investigated. The optimum condition were 7 mM of ammonium concentration, 1.94 mM of phosphate concentration and pH value of 8 with maximum hydrogen yield of 2784.73 ppm. Moreover it was determined that the microalgae grows best with 0.25% (v/v) of sodium carbonate added into the growth medium as additional carbon source. **Novelty/Improvements:** Optimization of the process of algae growth and hydrogen production with different levels was achieved using the RSM Box-Behnken design experiment.

Keywords: Algal Growth Rate, Ammonium, Bio-Hydrogen, Box-Behnken Design, pH, Phosphate, Response Surface Method (RSM), Sodium Bicarbonate, Sulfate

1. Introduction

Global energy supply around the world especially in developing countries has always been dependent fossil fuels. Fossil fuels are not carbon neutral and upon combustion releases green- house gases; hence, causing negative impacts on the environment. Besides the greenhouse gases (mainly carbon dioxide and methane), combustion of fossil fuel also results in the formation of compounds such as CO_X, NO_X, SO_X, C_XH_y, ash and sooth which are released into the atmosphere; and would eventually lead to global warming, climate change, environmental degradation and health problems[1,2]. Hydrogen is known as fuel of the future due to its high energy density per unit weight, 122 kJ/g which is higher than hydrogen carbon fuel and because its usage produces only water as a byproduct[3]. Hydrogen, like electricity, is an energy carrier rather than an energy resource. The keen interest in hydrogen as an alternative fuel is due to its ability to power fuel cells in zero-emission electric vehicles, its potential for domestic production, and the fuel cell's potential for high efficiency. However currently 99% of the total hydrogen is produced by non-renewable technologies which are through steam reformation of natural gas (~50%), petroleum refining (~30%) or gasification of coal (~20%)[2]. These conventional methods of producing hydrogen are energy intensive and are not environmentally friendly. Hydrogen can also be produced from water through electrolysis, but this method is much more energy intensive. Bio-hydrogen production by different microorganisms represents a far

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more promising approach due to the renewable, low-cost and environmentally friendly nature of this process[4]. In addition, certain methods of bio-hydrogen production, such as Microbial Electrolysis Cells (MEC), dark- and photo-fermentation, as well as algal photo heterotrophic biodegradation can utilize various low-priced industrial and agricultural wastes, therefore, coupling organic waste treatment with renewable energy generation[5]. Hydrogen can be generated from sustainable domestic and renewable energy resources, such as wind or solar-powered electrolysis, which will enhance the long term energy security[6]. Bio-hydrogen production by living organism is environmentally friendly and can be operated at ambient temperature and pressure with minimal energy consumption. Nevertheless this process still requires further improvement to be economically viable.

Aerobic green algae (eukaryotes), cyanobacteria (blue-green algae) and anaerobic photosynthetic bacteria (gram negative prokaryotes) are able to produce hydrogen through direct: 1. bio-photolysis, 2. indirect bio-photolysis, 3. photo-fermentation, and 4. dark fermentation[7]. Generally direct bio-photolysis involves the splitting of water molecule into proton (H^+) and oxygen in the presence of sunlight. The proton will then be converted into hydrogen catalyzed by the enzyme hydrogenase which is a hydrogen producing enzyme which uses ferredoxin as its primary electron donor[8,9]. However, there are some challenges limiting bio-hydrogen production by microalgae, ranging from the O_2 sensitivity of algal hydrogenase and the competition for a photosynthetic reducing agent-ferrodoxin to inefficiencies in the utilization of solar light energy[10]. During bio-photolysis hydrogen and oxygen co-evolve together, and when a certain amount of oxygen is present it will inhibit the activity of hydrogenase and stops it from producing hydrogen. The past decade brought the promise of surpassing these shortcomings through a series of significant breakthroughs. The two stage bio-photolysis process was developed. This allows the temporal separation of the oxygenic photosynthesis and photo-biological hydrogen production[11]. The process is achieved by deprivation of sulfur in the medium. The first stage is usually the cultivating of algae cells in sulfur rich medium to promote high rate of photosynthesis hence leading to high growth rate. After sufficient growth it is then transferred into sulfur deprived medium. Sulfur deprivation leads to a reversible decline on the rate of oxygenic photosynthesis but there is no effect on the rate of mitochondrial respiration[12]. The culture then becomes anaerobic and with the presence of light the enzyme hydrogenase is activated leading to active production of hydrogen gas[13]. This process is reversible therefore the algae cells are able to be cycled between the oxygen production phase and hydrogen production phase. In addition, it was showed that sulfur deprivation further enhanced the bio-hydrogen production of *Chlorella protothecoides* under nitrogen limitation culture condition[14]. Besides that, the effects of pH, nutrient concentration, and light intensity on the production of bio-hydrogen have also been studied under sulfur deprived conditions[15-17].

Biological hydrogen is currently more expensive than other fuels. Better understanding of the key parameters than influence the yield of hydrogen can help in bringing down the costs. The optimization of key experimental factors and genetic modification can ultimately improve the yield to make hydrogen production cost-effective and sustainable. In this study the microalgae, *Chlamydomonas reinhardtii* was used as the model algae to study the effects of nutrient concentrations, pH, and sodium carbonate concentration and microwave irradiation on the growth and the hydrogen production rate. Low doses of microwave irradiation have been hypothesized to be able to bring about improvement on yield of some valuable products in some microorganism. Specifically the effects of concentration of ammonium and phosphate and pH value of the medium on the hydrogen production by the microalgae, *C. reinhardtii* were studied. Optimization of nutrient concentrations of ammonium and phosphate and pH in the medium under sulfur deprived conditions for maximum hydrogen production was performed using Response Surface Methodology (RSM). The Box-Behnken design was performed to calculate the mathematical relationship linking the variables and its response. Studies on the effect of various concentration of sodium carbonate on the growth and effect of microwave irradiation on both microalgae growth rate and bio-hydrogen yield were also carried out.

2. Methodology

2.1 Materials

Chlamydomonas reinhardtii C137(+) (20mL) was obtained from Culture Collection of Algae and Protozoa (CCAP) KH of Scottish Association for Marine Science (SAMS) Limited in United Kingdom and its strain number is CCAP 11/32A. The microalgae were cultivated in Tris-Acetate-Phosphate (TAP) growth medium.

The growth medium used was TAP medium. TAP medium is widely used in researches as it can provide extra carbon source that allows *Chlamydomonas* cells to develop rapidly. The composition of the TAP stock solution is shown in Table 1. All the chemicals used in the growth medium were of analytical grade. All stock solutions were stored in the refrigerator.

To make the final TAP medium 2.42 g of Tris, 25 mL of TAP salt, 0.375 mL of phosphate solution, 1.0 mL Hutner's Trace element and 1.0 mL of glacial acetic acid was mixed in ~600 mL of RO water. Once all is dissolved the volume was made up to 1L by topping up with RO water.

Sulfur-free TAP (known as TAP-S) medium for hydrogen generation of *C. reinhardtii* was prepared in the similar way as the TAP medium but the sulfate salts were replaced with equimolar of chloride salts. In sulfur free TAP salt, $MgSO_4.7H_2O$ was replaced with $MgCl_2.H_2O$. Where as in the Hutner's trace element, $ZnSO_4.7H_2O$ was replaced with $ZnCl_2$ (1.0 g), $CuSO4.5H_2O$ was replaced with $CuCl_2.2H_2O$ (0.1 g) and $FeSO_4.7H_2O$ was replaced with $FeCl_2.4H_2O$ (0.36 g). The final TAP-S medium was the same as TAP medium.

2.2 Methods

2.2.1 Cultivation of Microalgae

Heterotrophic algae cultivation technique, which is extensively used, was used to cultivate the algal cells. Heterotrophic cultivation can provide high cell density, and high specific growth rate[18]. The algae cultures were grown using a 250 mL Schott bottle containing 150 mL of TAP growth medium. Fifteen milliliters (mL) of *C. reinhardtii* were cultivated in the TAP growth medium as prepared previously. The cells were left to be cultivated for 4 days at a temperature between 25 to 28°C. The algae cultures were illuminated continuously from the top using cool white fluorescents light with intensity 2500 lux measured using a lux meter. Distance between the algae suspension and fluorescent light was 1 m which was sufficient to avoid photo inhibition. The microalgae cultures were hand shaken once or twice daily to avoid the adherence of the microalgae to sides of the culture bottles. Cell density was measured daily for 6 days using spectrophotometer (Optizen Pop UV/Vis Spectrophotometer-Korea) at wavelength of 750 nm.

2.2.2 Effect of Sulfur on Microalgae Growth Rate

Algae were cultivated in TAP medium and TAP-S medium respectively. The cultivation procedures were similar with the procedure in cultivation of microalgae. For the cultivation of algae in TAP-S medium, the algae suspension was first centrifuge (Hettich Rotofix 32 bench top centrifuge-Germany) at 2000 rpm for 20 minutes to remove the TAP medium. The algae pellet was then re suspended with TAP-S medium. The algae cells were continuously illuminated by cool white fluorescents light at an average of 2500 lux under room temperature. As previously, the cell density was measured daily for six days consecutively.

2.2.3 Optimization of Microalgae Growth Rate in TAP Medium

To determine the optimal condition for the algae growth rate, three parameters in the TAP growth medium was altered. The three parameters were ammonium, phosphate and sulphate. For each of the parameters, three levels of vari-

Table 1. TAP stock solution composition

Stock Solutions	Compound	Amount (gram)	Water (mL)	
TAP Salt	NH_4Cl $MgSO_4.7H_2O$ $CaCl_2.2H_2O$	15.0 g 4.0 g 2.0 g	Dissolve in ~850mL of RO water, once all is dissolved top up to 1L.	
Phosphate Solution	K_2HPO_4 KH_2PO_4	28.8 g 14.4 g	Dissolve in ~70mL of RO water, once all is dissolved top up 100mL.	
Hutner's Trace Element	EDTA $ZnSO_4.7H_2O$ H_3BO_3 $MnCl_2.4H_2O$ $CoCl_2.6H_2O$ $CuSO_4.5H_2O$ $(NH_4)_6MO_7O_{24}.4H_2O$ $FeSO_4.7H_2O$	5.0 g 2.2 g 1.14 g 0.50 g 0.16 g 0.16 g 0.11 g 0.50 g	25 mL 10 mL 20 mL 5 mL 5 mL 5 mL 5 mL 5 mL	Top up to 1L of RO water

ables (low level, medium level, high level) were optimized, as shown in Table 2. Note that the low level concentration for every parameter is 1/10 of the medium level and the high level concentration is 1.9 times the medium level. The values in the parenthesis represent the coded values of the software. The ammonium, phosphate and sulfate concentrations were 7 mM, 1.02 mM and 1 mM respectively in a common TAP medium, which also represents as the center point of this experiment. This center point was then repeated three times to evaluate the experimental errors.

Based on the Design Expert 9.0.6.2 software (Stat-Ease, Minneapolis, USA), the setting conditions for 15 experimental run were generated. The respective culture medium was then prepared according to that particular setting condition. After that, five mL of algae cells were taken from the algae culture suspension and were harvested at 3500 rpm for 15 minutes by centrifugation. The cell pellet were then washed two times using the modified TAP medium before re suspending it into 50 mL of the particular modified TAP medium in a 250 mL Schott glass bottle. The algae cells were continuously illuminated by cool white fluorescents light at an average of 2500 lux under room temperature. Cell density was measured daily for six days consecutively. The average growth rate during the exponential stage of algal growth was then calculated using following Equation: $C_t = C_o \times e^{\mu t}$; where C_t and C_o are the cell concentrations at time t and 0 correspondingly and μ is the specific growth rate.

After the results of average growth rate was obtained based on the experimental works, the optimization study was performed by using Box-Behnken Design[19].

2.2.4 Optimization of Bio Hydrogen Yield under Sulfur Deprived Condition

This experiment was conducted to determine the best optimized TAP-S medium to obtain the highest bio hydrogen yield. Three parameters in the TAP-S medium were altered, which are the ammonium concentration, phosphate concentration and pH of the medium (sulfate concentration was kept zero, and a one new parameter which was pH was added). For each of the parameters, three levels of variables (low level, medium level and high level) were optimized, as shown on Table 3.

Forty mL of algae in it mid-logarithmic phase (OD_{750nm}= 0.81) was obtained from the pre-cultivation (normal growth under TAP-medium) suspension and was harvested by centrifugation at 3500 rpm for 20 minutes. After that, the wet cell pellet were then washed twice with the particular TAP-S medium before suspending into 2 L Schott bottle with stirrer containing 400 mL of the modified TAP-S medium. The microalgae culture was left at room temperature with continuous white fluorescents light at 2500 lux. After 96 hours the amount of bio-hydrogen produced by the algae was measured. Ten mL of the gas was extracted from the headspace of the glass bottle using a gas tight syringe. Hydrogen gas concentration was measure using the hydrogen gas meter. For the optimization study, the corresponding bio-hydrogen yields for six types different culture medium was performed as recorded in the result section. This experiment was then modeled in the Design Expert software by using RSM-Box-Behnken Design.

2.2.5 Cultivation of Microalgae in Growth Media Containing Variable Amount of Sodium Bicarbonate

To study the effect of addition of sodium bicarbonate on the growth of microalgae, the algae cells were grown at different concentrations of sodium bicarbonate in TAP growth medium. The concentrations of sodium bicarbonate were 0% $NaHCO_3$, 0.25% $NaHCO_3$, 0.50% $NaHCO_3$, 1% $NaHCO_3$ and 3% $NaHCO_3$. Cell growth and biomass

Table 2. Level of variables chosen for microalgae growth rate

	Ammonium (mM), X1	Phosphate (mM), X2	Sulfate (mM), X3
Low level (L) (-1)	0.70	0.10	0.10
Medium level (M) (0)	7.00	1.02	1.00
High level (H) (+1)	13.30	1.94	1.90

Table 3. Level of variables chosen for bio hydrogen production by microalgae

	Ammonium (mM), X1	Phosphate (mM), X2	pH value, X3
Low level (L) (-1)	0.70	0.10	6
Medium level (M) (0)	7.00	1.02	7
High level (H) (+1)	13.30	1.94	8

weight were measured daily for 8 days of the growth period.

2.3 Analytical Method

2.3.1 Measurement of Algae Growth Rate

The growth of microalgae was determined in terms of an increase in Optical Density (OD) and biomass was estimated in terms of dry weight. OD of the algae was measured using Optizen Pop UV/Vis Spectrophotometer (Korea) at wavelength of 750 nanometres (nm). Wavelength of 750 nm was chosen as it will not interfere with the chlorophyll absorbance. Before measuring the OD of the algae, TAP medium was used as a blank to zero the spectrophotometer. Four mL of algae were withdrawn from the culture suspension and inserted into a cuvette to measure the OD. To obtain the standard curve for the algae growth, 5 serial dilutions of algae was carried out and the OD at 750 nm of each dilution were measured.

Dry weight of microalgae was measured by filtering 10 mL of the diluted algae (after the measurement of the OD) on a pre-weighted filter paper. The filter paper was then dried at 70°C in the oven overnight. After 24 hours of drying the weight of the filter paper was measured using an electronic analytical balance. The dry weight of algal biomass was obtained and was corresponded with the OD obtained earlier to establish a relationship. Subsequent dry biomass of the algae from various experiments was measured using this relationship.

2.3.2 Measurement of Bio-Hydrogen Production Yield

The bio-hydrogen yield was determined by collected the gas produced at the headspace of the Schott bottle using a gas tight syringe. A 10 mL aliquot of the gas sample was collected and injected into the hydrogen meter (Crowcon gas meter). The amount of hydrogen gas detected in the gas sample (in ppm) was multiplied by the total headspace in the bottle to obtain total hydrogen gas produced.

3. Results and Discussion

3.1 Cultivation of Microalgae

Standard curve of algae growth was obtained to show the relationship between the biomass and optical density at 750 nm. The biomass of the algae increases linearly with increase of its OD. This is expected as the OD increases more light is absorbed by the cells in the sample leading to higher biomass production[20]. From the graph, the best fit line obtained to determine an equation which will then be used to get the biomass of algae for the later experiments.

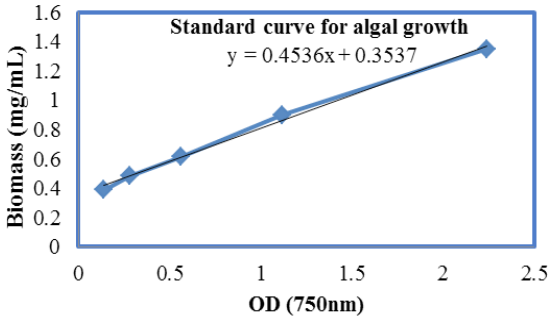

Figure 1. Standard curve for *C. reinhardtii* growth measured at 750 nm to obtain the biomass relationship with OD

Dry Algal Biomass (mg/mL) = 0.4536 x OD_{750nm} + 0.3537

3.2 Growth of Microalgae in TAP and TAP-S Medium

As can be observed in Figure 1, the difference between the growth of microalgae in TAP and TAP-S medium is clearly observed. Algae growth in TAP medium producers a higher graph slope compared to algae growth in TAP-S medium, which indicates a better growth rate. Based on Figure 2, for the growth in TAP medium the algal cells went through the exponential stage from 0th hour to 48th hour. Therefore, the average growth rate can be calculated using Equation (1) below:

$$C_{48h} = C_{0h} \times e^{\mu(48-0)} \quad (1)$$

$$0.851 = 0.403 \times e^{\mu x 48}$$

$$\mu_{average} = 0.0152/h$$

Thus the maximum specific growth rate was 0.015/h

Moreover algae growth in TAP-S medium reaches the death phase much earlier which is at about 120th hours. This could possibly due to nutrient starvation as TAP-S medium lacks sulphur which is an important nutrient for cell growth and photosynthesis. Sulfur is required for algae growth as it will be reduced into sulphide and incorporated into cysteine which is the key intermediate from which most of the sulphur compounds are synthesized for algae growth[21]. Hence this result in the inability of algae in

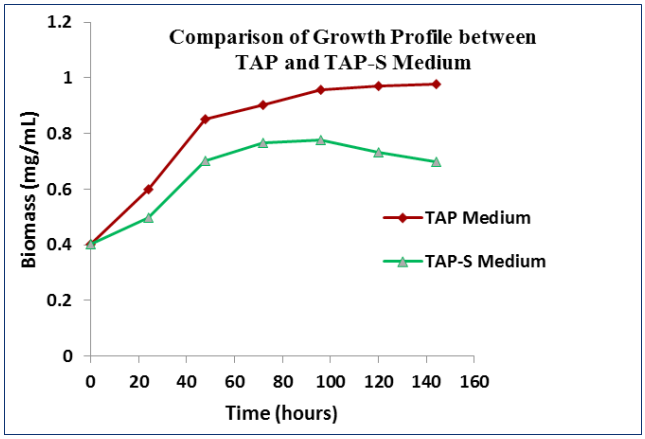

Figure 2. *C. reinhardtii* was cultivated in TAP and TAP-S medium. The OD at 750nm was measured at different time intervals and the biomass dry weights were estimated from the standard graph of OD 750nm vs dry biomass

TAP-S medium to sustain active photosynthesis as sulfur is an essential element for photosynthesis. When sulfur is present, microalgae are able to reduce sulfur to sulphide and incorporate it into cysteine. Cysteine is the central intermediate from which most of the sulfur compounds are synthesized.

3.3 Optimization TAP Medium for Enhanced Microalgae Growth Rate

Based on the daily OD_{750nm} of the algae grown in the six types of different culture medium, the average growth rate was calculated and recorded to be used for the analysis using Design Expert software (Box-Behnken Design). According to the results obtain the stationary phase for all the algae culture occurred during the first 48 hours. Based on the design, fifteen experimental runs were required to be carried out in order to maximize the algal growth. Table 4 shows the experimental average growth rate for different culture medium used. The average growth rate was calculated based on the biomass during this period by using Equation (1). The predicted growth rate was generated using the RSM-Box Behnken Design software.

Table 5 shows the fit summary table, one of the several useful statistical tables provided by Design Expert to determine which model to select for further study. Based

Table 4. Experimental average growth rate in different culture medium and predicted algal growth using RSM-Box Behnken Design

Trial	Ammonium Concentration (mM)	Phosphate Concentration (mM)	Sulfate Concentration (mM)	Experimental average growth rate (/h)	Predicted average growth rate (/h)
1	0.7	0.10	1.0	0.00893	0.00881
2	13.3	0.10	1.0	0.01061	0.0116
3	0.7	1.94	1.0	0.00812	0.00916
4	13.3	1.94	1.0	0.01197	0.01195
5	0.7	1.02	0.1	0.00696	0.00799
6	13.3	1.02	0.1	0.01111	0.01078
7	0.7	1.02	1.9	0.01191	0.00997
8	13.3	1.02	1.9	0.01339	0.01276
9	7.0	0.10	0.1	0.01497	0.01429
10	7.0	1.94	0.1	0.01544	0.01464
11	7.0	0.10	1.9	0.01535	0.01626
12	7.0	1.94	1.9	0.01573	0.01661
13a	7.0	1.02	1.0	0.01529	0.01545
14a	7.0	1.02	1.0	0.01555	0.01545
15a	7.0	1.02	1.0	0.01581	0.01545

[a] The center point of this experiment was replicated three times.

Table 5. Fit summary table for algal average growth rate

Source	Sequential p-value	Lack of Fit p-value	Adjusted R-Squared	Predicted R-Squared	Remarks
Linear	0.5125	0.0057	-0.0415	0.00893	
2FI	0.971	0.10	-0.3923	0.01061	
Quadratic	0.0013	0.0365	0.881	0.3341	Suggested
Cubic	0.0365		0.9927	0.01197	Aliased

on the table, quadratic model is recommended as it has the smallest sequential p-value.

Ideally, the best model should have an insignificant probability value (P>0.10). In this case, the highest lack-of-fit p-value was selected by the software. The adjusted and predicted R-squared are defined as the amount of variation that can be explained by the model. The higher the R-squared values, the better the model. Thus, the quadratic model with the highest R-squared values was selected.

The ANOVA (analysis of variance) table was generated by the software and this table plays a significant role to test the hypothesis on the parameters of the model. A larger F value indicates that the variance contributed by the model is significantly larger than random error. Based on the Table 6, the term "Model" has the highest F-value (29.08) which implies that the suggested model is significant. Moreover there is only a 0.01% chance that the F-value occurred due to noise. The value for "Prob > F" for every model term is suggested to be less than 0.05, hence A, C and A^2, are the significant model terms. To check weather all the data points were predicted by the model chosen, the graph for relationship between actual response and predicted response was plotted. As can been seen from the graph (Figure 3), the data points are distributed evenly on a 45° line which indicated that the modified model selected is capable of predicting all of the date points.

By examining all of the results above, it can be concluded that the modified quadratic model was sufficient to describe the response surface within this region and represented the data with accuracy in the experimental region. The predicted average growth rate equation is as follows:

$$\text{Predicted average growth rate} = 0.00634366 + 0.00201105 \times \text{Ammonium Concentration} + 0.000190217 \times \text{Phosphate Concentration} + 0.00109722 \times \text{Sulfate Concentration} - 0.00012$$

The predicted average growth rate was then used as the response during the optimization analysis which would provide a higher degree of accuracy as compared to the experimental rate. To maximize the predicted average growth rate, constraints were set according to values as shown in Table 7.

From the 100 of solutions proposed by the software, the combination with the lowest average nutrient concentration, i.e. 7.2 mM of ammonium concentration, 1.81 mM of phosphate concentration and 1.89 mM of sulfate concentration was selected as the final optimal condition needed to obtain a maximum predicted growth rate.

The perturbation plot was plotted to compare the effect of all the variables on the algal average growth rate at its optimal condition. It is a "one-factor-at-a-time" investigation and is useful in determining the individual effect of several factors on the response of the experiment. As shown in Figure 4, ammonium concentration is the most significant parameters among the other two, as slight changes in the ammonia concentration will cause major difference in the response of the experiment. As the ammonium concentration increases, the predicted average growth rate increases until a maximum point at 0.01662/h and beyond this point any further increase in its concentration would lead to a decrease in the algal growth rate. Both phosphate and sulfate concentration predicted growth rate increases linearly, however sulfate concentration has a steeper slope indicating that the algae growth rate is more sensitive to sulfate than phosphate concentration.

The interactions between factors could not be demonstrated by this perturbation plot (Figure 4). For that

Table 6. ANOVA table for algal average growth rate

Source	Sum of Squares	dof	Mean Square	F Value	p-value (Prob > F)	Remarks
Model	1.20E-04	4	2.99E-05	29.08	< 0.0001	significant
Aa	1.56E-05	1	1.56E-05	15.13	0.003	
Bb	2.45E-07	1	2.45E-07	0.24	0.6361	
Cc	7.80E-06	1	7.80E-06	7.58	0.0204	
A2	9.61E-05	1	9.61E-05	93.38	< 0.0001	
Residual	1.03E-05	10	1.03E-06			
Lack of Fit	1.02E-05	8	1.27E-06	18.78	0.0515	not significant
Pure Error	1.35E-07	2	6.76E-08			
Cor Total	1.30E-04	14				

[a]A represents the ammonium concentration. [b] B indicates the phosphate concentration. [c] C symbolizes the sulfate concentration.

Table 7. Constraints for the optimization of algal average growth rate

Name	Goal	Lower Limit	Upper Limit	Lower Weight	Upper Weight	Importance
A: Ammonium Concentration	is in range	0.7	13.3	1	1	3
B: Phosphate Concentration	is in range	0.1	1.94	1	1	3
C: Sulfate Concentration	is in range	0.1	1.9	1	1	3
Predicted average growth rate	maximize	0.007992	0.01661	1	1	3

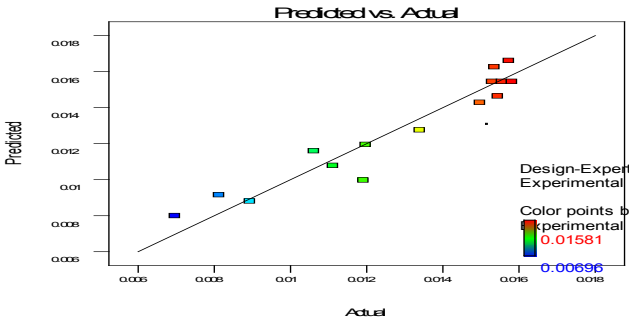

Figure 3. Relationship between predicted and experimental response for algal average growth rate

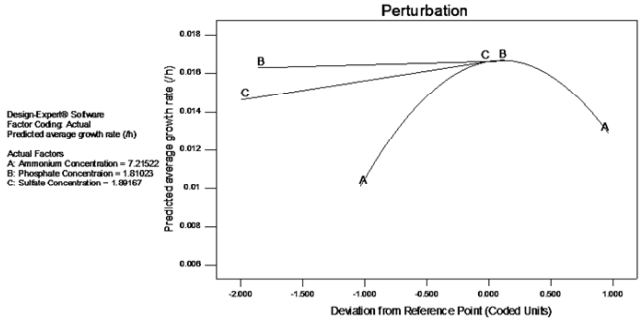

Figure 4. Perturbation plot for algal average growth rate

purpose, Figure 5, which is a three-dimensional response, indicates interaction between factors towards the average algal growth. The plot (Figure 5) shows how ammonium and sulfate concentration affect the algal growth rate. Phosphate concentration in this plot is held constant as it has minor effect on the algal growth rate. In conclusion, through the optimization study using RSM Box-Behnken Design, Design Expert software predicted that 7.22 mM of ammonium concentration, 1.81 mM of phosphate concentration and 1.89 mM of sulfate concentration will give a maximized algal growth rate of 0.01662 /h.

3.4 Cultivation of Microalgae in Growth Containing Variable Amount of Sodium Bicarbonate

Carbon is an important source for microalgae growth as photosynthesis requires carbon. Hence in general,

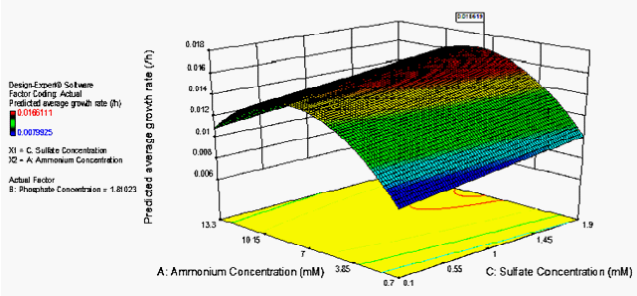

Figure 5. Three-dimensional response for the algal average growth rate

the more carbon supplied to the algae, the algae growth rate increases. However as seen on Figure 6 only the culture with 0.25% and 0.5 % sodium bicarbonate showed higher rate of growth; while the rest (1% and 3%) showed decrease in growth rate. The addition of 0.25% (v/v) of sodium bicarbonate increased the algae growth rate but the algae growth had a longer lag phase compared to the algae grown in 0% sodium bicarbonate. Although its lag phase was longer, the algae growth accelerates tremendously after the lag phase.

A reason for this could be that the amount of carbon (in the form of acetate) supplied in the cultivation media was of sufficient quantity for the growth of the algae and any more carbon supplied will have an overdose effect which could decrease its growth rate. Only the addition of 0.25% (v/v) of sodium bicarbonate further increased the algae growth rate but the algae has a longer lag phase compared to the algae grown in 0% sodium bicarbonate. Although its lag phase was longer, the algae growth accelerates tremendously after the lag phase. Algae grown in TAP medium containing 3% (v/v) of sodium bicarbonate showed almost negligible growth within the experimental period. Another possible reason could be that, *C. reinhardtii* has an optimum growth at pH 7.5, and addition of sodium bicarbonate could alter the pH and affect the growth rate[22]. Although after sodium bicarbonate was added into TAP growth medium, its pH was adjusted to 7.5, however after a few days the pH of the growth medium will eventually increase affecting the solubility of some nutrient affecting its growth[23]. Moreover when high carbon dioxide concentration is present in the medium, the microalgae cells will reach anaerobiosis earlier as it makes the algae cells to consume all the available sulfur[24]. Therefore instead of undergoing to photosynthesis to promote further growth, the algae cells will be undergoing hydrogen production mode[25].

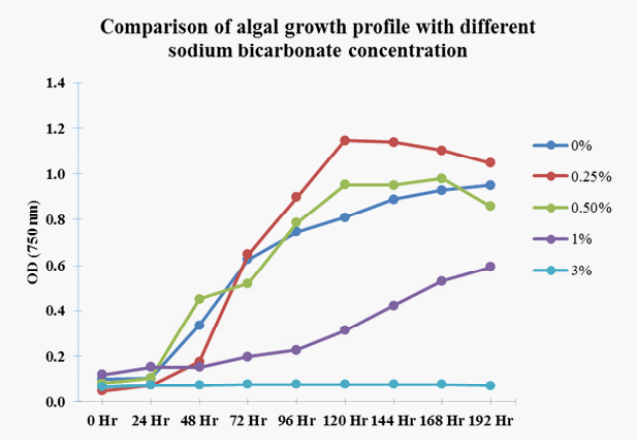

Figure 6. C. *reinhardtii* strain was cultivated in TAP medium with sodium carbonate concentration of 0%, 0.25%, 0.50%, 1% and 3% (v/v). The OD at 750 nm was measured at different intervals.

3.5 Optimization of Biohydrogen Yield under Sulfur Deprived Condition

Table 8 shows the optimization results for maximum yield of bio-hydrogen under conditions of variation of ammonium concentration, phosphate concentration and pH in the TAP-S culture media. Results from this experiment were then modelled in the Design Expert software by using RSM-Box-Behnken Design. The number of the experimental runs and their corresponding setting conditions are summarized in Table 8.

As mentioned previously, there are several statistical tables provided by Design Expert software in order to check the appropriateness of the model selected. Table 9 and 10 are some of the useful statistical summaries which verify that the modified quadratic model chosen is well enough to illustrate the pattern of experimental bio-hydrogen yield.

As mentioned previously, the larger the F value, the more likely the variance contributed by the model is significantly larger than random error. The "model" has a F-value of 18.56 which implies that the suggested model is significant. A, B and C are the significant model terms as their "Prob>F" values are lesser than 0.05. To check weather all the data points were predicted by the model chosen, the graph for relationship between actual response and predicted response was plotted. As can been seen from the graph (Figure 7), the data points are distributed evenly on a 45° line which indicated that the modified model selected is capable of predicting all of the date points.

Table 8. Optimization study of bio-hydrogen yield using RSM-Box Behnken Design

Trial	Ammonium Concentration (mM)	Phosphate Concentration (mM)	pH	Biohydrogen yield (ppm)	Predicted Biohydrogen yield (ppm)
1	0.7	0.10	7	1450	1219.02
2	13.3	0.10	7	1570	1638.02
3	0.7	1.94	7	1690	1696.52
4	13.3	1.94	7	2240	2115.52
5	0.7	1.02	6	1594	1621.98
6	13.3	1.02	6	1600	1543.98
7	0.7	1.02	8	1500	1696.48
8	13.3	1.02	8	2500	2612.48
9	7.0	0.10	6	1640	1735.73
10	7.0	1.94	6	2000	2213.23
11	7.0	0.10	8	2240	2307.23
12	7.0	1.94	8	2880	2784.73
13[a]	7.0	1.02	7	2560	2461.69
14[a]	7.0	1.02	7	2551	2461.69
15[a]	7.0	1.02	7	2555	2461.69

[a] The center point of this experiment was replicated three times.

Table 9. ANOVA table for biohydrogen yield

Source	Sum of Squares	Dof	Mean Square	F Value	p-value (Prob > F)	Remarks
Model	3.11E+06	6	5.18E+05	18.56	0.0003	significant
A-Ammonium Concentration	3.51E+05	1	3.51E+05	12.58	0.0075	
B-Phosphate Concentration	4.56E+05	1	4.56E+05	16.34	0.0037	
C-pH	6.53E+05	1	6.53E+05	23.41	0.0013	
AC	2.47E+05	1	2.47E+05	8.85	0.0177	
A^2	1.31E+06	1	1.31E+06	46.79	0.0001	
B^2	1.51E+05	1	1.51E+05	5.4	0.0486	
Residual	2.23E+05	8	27908.62			
Lack of Fit	2.23E+05	6	37204.71	1829.74	0.0005	significant
Pure Error	40.67	2	20.33			
Cor Total	3.33E+06	14				

Based on Figure 7 the data points are distributed evenly on 45° line, indicating the model selected is capable of predicting all of the data points.

By analysing the results above it is proven that the modified quadratic model is sufficient to illustrate the pattern of experimental bio-hydrogen yield using the following equation:

$$\text{Predicted biohydrogen yield} = 917.0522 - 33.69963 \times \text{Ammonium Concentation} + 745.07507 \times \text{Phosphate Concentration} + 9.63889 \times \text{pH} + 39.44444 \times \text{Ammonium Concentration}$$

The predicted bio-hydrogen yield is then calculated and recorded in the last column of Table 8.

In order to run the optimization analysis, some constraints were set as shown in Table 10. The predicted bio-hydrogen yield was then used as the response during the optimization analysis which would provide a higher degree of accuracy as compared to the experimental date. To maximize the predicted average growth rate, constraints were set according to values as shown in Table 1.

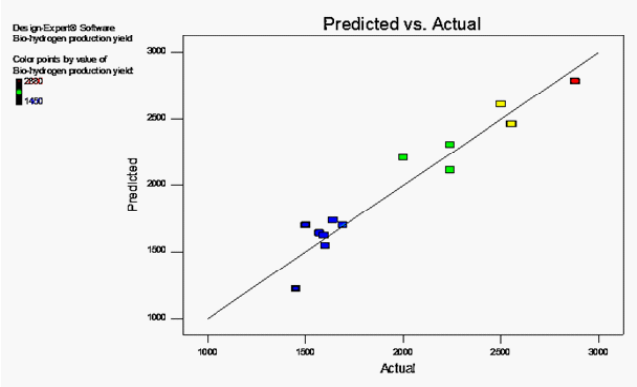

Figure 7: Perturbation plot for algal average hydrogen production rate

From the 100 of solutions proposed by the software, the combination with the lowest average nutrient concentration, i.e. 7 mM of ammonium concentration, 1.94 mM of phosphate concentration and pH value of 8 was selected as the final optimal condition needed to obtain a maximum bio-hydrogen yield.

Based on the Figure 8, A, B and C indicates the individual effect of ammonium concentration, phosphate concentration and pH on the yield of bio-hydrogen. The perturbation plot shows that the ammonium and phosphate concentration has a bell-shaped effect on the predicted bio-hydrogen yield and the pH value has a linear relationship with the response. This shows that the bio-hydrogen yield is sensitive to the interactions between the ammonium and phosphate concentrations.

To illustrate the interaction between these factors, three-dimensional response was plotted as shown in Figure 9. Based on the plot, it is observed that the optimum point is located inside the design boundary. The peak of the graph shown in Figure 9 represents the optimal point predicted by the software after the optimization analysis. Hence the optimal conditions to maximize the amount of bio-hydrogen produced by the algae, *Chlamydomonas reinhardtii* under sulfur-deprived condition are 7 mM of ammonium concentration, 1.94 mM of phosphate concentration and pH value of 8 with optimized bio-hydrogen yield of 2784.73 ppm.

3.6 Verification of Optimized TAP and TAP-S Culture Medium

The predicted optimized response for both algal growth rate and bio-hydrogen yield were verified by conducting another set of experiments. Based on Figure 10, algae grown in optimized TAP medium has the steepest slope between 0th and 48th hours and it has the best average

Table 10. Constraints for the optimization of biohydrogen yield

Name	Goal	Lower Limit	Upper Limit	Lower Weight	Upper Weight	Importance
A: Ammonium Concentration	is in range	0.7	13.3	1	1	3
B: Phosphate Concentration	is in range	0.1	1.94	1	1	3
C: pH	is in range	6	8	1	1	3
Predicted biohydrogen yield	maximize	1219.02	2784.73	1	1	3

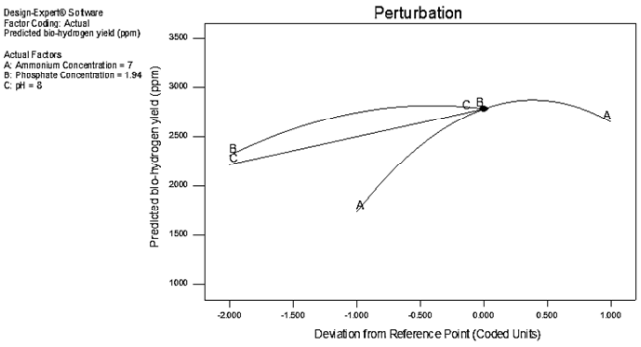

Figure 8: Perturbation plot for bio-hydrogen yield

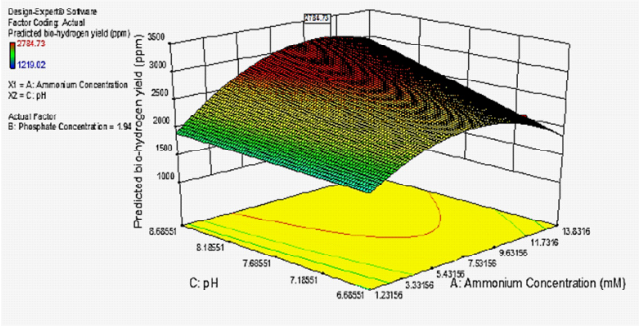

Figure 9: Three-dimensional response for biohydrogen yield

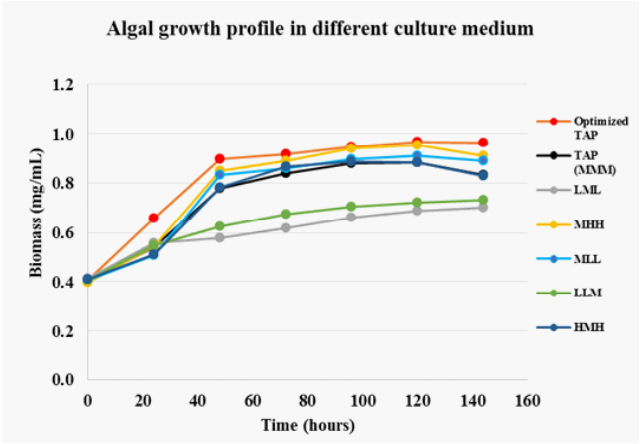

Figure 10: Algal growth profile in different culture medium

growth rate of 0.0165/h. Although the value of experimental growth rate is lower than the predicted growth rate calculated by the software, it is still higher than the highest experimental value (0.01573/h) obtained for algae culture in *Medizinische Hochschule Hannover* (MHH) culture medium. This validates the effectiveness of the optimization studies using Box-Behnken Design.

As for the bio-hydrogen yield, the optimized TAP-S medium produced 2880 ppm of bio-hydrogen. The experimental bio-hydrogen yield is higher than the predicted value (2784.73 ppm) generated by the software and this verifies the predicted optimized result from the software.

4. Conclusion

Based on the outcomes of this research, it is concluded that the green algae, *Chlamydomonas reinhardtii* is best grown in sulfur rich medium as absence of sulfur reduces its growth rate by 10.33%. Algae grown in medium lacking sulfur deprived medium consumes more nutrients leading to early death phase due to nutrient limitations. However, sulfur deprived medium is required for the production of bio-hydrogen. Optimization of the process of algae growth and hydrogen production with different levels was achieved using the RSM Box-Behnken design experiment. By conducting minimal experiments, the optimal conditions were obtained and the responses were described with a mathematical equation. The Design Expert 9.0.6.2 predicted that 7.22 mM of ammonium concentration, 1.81 mM of phosphate concentration and 1.89 mM of sulfate concentration will provide an optimized algal growth rate of 0.016619 /h. On the other hand, optimized biohydrogen yield of 2784.73 ppm can be obtained by cultivating in the sulfur-deprived medium with 7 mM of ammonium concentration, 1.94 mM of phosphate concentration and pH 8. These predictions were then verified by another set of experiments. Lastly, growing microalgae with excessive addition of carbon source (sodium bicarbonate) hinders the growth of the microalgae. The microalgae grow more effectively with only an addition of 0.25% (v/v) sodium bicarbonate into the TAP growth medium.

Some future work can be carried out to further improve the present findings. Other than the nutrient concentration of the culture medium, variables such as light intensity, type of buffer solution, and surrounding temperature should be added in to completely study the effect of every variable on the algal growth rate and bio-hydrogen yield. Then, based on the ranking of the significance of the variables, three or more variables can be selected to optimize the conditions in order to maximize the algal growth rate and bio-hydrogen yield separately. There is also possibility to maximize the bio-hydrogen yield and algal growth rate at the same time by using RSM Box-Behnken Design by setting the algal growth rate

and bio-hydrogen yield as the response 1 and response 2 respectively in the software.

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