International Journal on
Advanced Science
Engineering
Information Technology

Vol.3 (2013) No. 2
ISSN: 2088-5334

Pre-Optimization Conditions for *Haematococcus pluvialis* Growth

Nurul Asmidar Hanan[a], Najeeb Kaid Al-Shorgani[ab], Hafiza Shukor[a], Norliza Abd. Rahman[a], Mohd Sahaid Kalil[a]

[a]*Department of Chemical and Process Engineering, Faculty of Engineering and Built Environment, Universiti Kebangsaan Malaysia, 43600 UKM Bangi, Selangor, Malaysia*
E-mail: asmidarhanan86@gmail.com, ieza_72@yahoo.com, liza@eng.ukm.my, sahaid@eng.ukm.my

[b]*School of Biosciences and Biotechnology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600 UKM Bangi, Selangor, Malaysia*
E-mail: nag_nas2001@yahoo.com

***Abstract*— The green microalgae *Haematococcus pluvialis* is one of the most beneficial microalgae due to its production of astaxanthin that has great commercial interest because of its antioxidant properties. A two level factorial design (2LFD) was used to pre-optimize conditions to grow *H. pluvialis*. The variables involved were pH, inoculum size, temperature and presence or absence of light. The results were analyzed by analysis of variance (ANOVA). ANOVA analysis showed that inoculum size of 40%, temperature of 30oC and presence of light had significant effects on *H. pluvialis* growth whereas production pH had insignificant effect. The established model from the ANOVA analysis had a significant value with *Pmodel* > F = 0.0074 and the R^2 value of 0.9989. The expected growth was 1.08879 (Table 5) and the optimized growth was 1.178 (Table 5).**

***Keywords*— *Haematococcus pluvialis* Growth; Pre-Optimization; Two Level Factorial Design.**

I. INTRODUCTION

Astaxanthin (3S,3S'-dihydroxy-β,β'-carotene-4,4'-dione) is a red-orange caratenoid pigment of high commercial interest due to its use in pharmaceutical properties. This caratenoid can enhance immune system by fighting cellular damage and prevent some cases of cancers (Gu. et al., 1997). Astaxanthin occurs in many aquatic animals such as salmon, trout, shrimp, red sea beam and many crustaceans (Johnson et. al, 1980). The increment in the demand for astaxanthin has led to an intense search for materials that are naturally rich in astaxanthin content. Haematooccus pluvialis , the fresh-water green unicellular alga has been identified as one of the most promising natural sources of astaxanthin.

In order to produce astaxanthin, the process involves two stages which are green stage which involves cultivation of green algae in order to increase its mass. The second stage is referred to carotegenesis stage. In this stage, green alga undergoes transformation to red algae after being exposed to some certain stress conditions (Kobayashi et al, 1997). *H. pluvialis* with the maximum mass followed by the second stage to produce astaxanthin (Kobayashi et al, 1997) under stress induction. Two stages production process have been proposed due to different culture conditions needed for production of green algae and astaxanthin accumulation (Olaizola, 2000). Algal growth related to productivity of astaxanthin (Jeon et. al, 2006). Therefore, this study focused on optimization productivities of *H. pluvialis* biomass so that can enhance productivities of astaxanthin by screening the effect of pH, inoculum size, temperature and presence of light using a Two Level Factorial Design (2LFD).

II. MATERIALS AND METHODS

A. Microorganism And Culture Conditions

Green algae, *H. pluvialis* was obtained from Algaetech Sdn. Bhd., Malaysia. All culture experiments were performed in 250 mL Scott Duran bottles containing 150 mL BBM medium in different incubator with different temperatures. Heterotrophic medium was selected by using sodium acetate, 0.25% (w/v) as carbon source (Orosa et.al, 2005). Sodium acetate, 0.25% (w/v) was used as carbon source in the experimental due to acetate was the best carbon sources, enhancing growth and carotegenesis (Kobayashi et. al, 1993). Gentle shaking by hand was carried out three times a day.

B. Media Preparation

Bold Basal Medium (BBM) was used as growing media for the culture of *H. pluvialis*. BBM consist of five elements

which are major stock solutions, boron stock solution, iron stock solution, trace metal stock solutions and alkaline EDTA stock solutions. The components of these solutions are presented in Table 1.

TABLE I
BBM COMPOSITIONS

Component	1 Liter Stock Solution
Major Stock Solution	
1. $NaNO_3$,	25 g
2. $CaCl_2$,	2.5 g
3. $MgSO_4.7H_2O$,	7.5 g
4. K_2HPO_4,	7.5 g
5. KH_2PO_4,	17.5 g
6. NaCl,	2.5 g
Boron Stock Solution	
H_3BO_3	11.42 g
Iron Stock Solution	
$FeSO_4.7H_2O$	4.98 g
Trace Metal Stock Solution	
1.$ZnSO_4.7H_2O$	8.82 g
2.$MnCl_2.4H_2O$	1.14 g
3.$(NH_4)6Mo_7O_{24}.4H_2O$	0.88 g
4.$CuSO_4.5H_2O$	1.57 g
5.$Co(NO_3)_2.6H_2O$	0.49 g
Alkaline EDTA Stock Solution	
1.EDTA	50 g
2.KOH	31g

C. Sampling Time

Samples were taken at 24 hours intervals for analysis for 20 days. The growth of the cell-biomass of samples taken was monitored by using Hach DR2800 spectrophotometer (Hach, Corolado, Canada) at 680nm (Kang et. al., 2005).

D. Experimental Design and Analysis of Data

2LFD was used to screen significant parameters affecting *H. pluvialis* growth. The experimental region extended from -1 to +1 in terms of each independent variable. Variables involved in the designs which were pH, inoculum size, temperature and presence of light coded as X1, X2, X3 and X4, respectively as showed in Table 2. According to the experimental design, 16 experiments were specified by the design and performed as shown in Table 3.

TABLE II
THE LEVEL OF VARIABLES

Code	Factor	Low Level (-1)	High Level (+1)
X1	pH	5	9
X2	Inoculum Size	5	40
X3	Temperature	15	30
X4	Presence of Light	-1 (Dark)	+1 (Light)

III. RESULTS AND DISCUSSION

The experimental results on the effect of pH, inoculum size, temperature and presence of light were analyzed using analysis of variance (ANOVA). Inoculum size, temperature and light had significant effect with $P \leqslant 0.05$ on *H. pluvialis* growth as indicated in Table 3. pH had insignificant effect to the growth with $P \geqslant 0.05$.

The most significant effect on *H. pluvialis* growth were inoculum size (P= 0.0010), light (P=0.0022) and followed by temperature (P= 0.0228). Interaction effect such as pH and inoculum size, temperature and light, pH, inoculum size and light were significant with $P \leqslant 0.05$. The interaction between pH and inoculum size shows that inoculum size can affect pH in order of *H. pluvialis* growth, similar goes to interaction of temperature and light, and interaction among pH, inoculums size and light. The fitted model used has high reliability with value Pmodel > F = 0.0074. The regression coefficient of determination (R^2) was 0.9989 indicated that 99.89% variables lead to response could be explained by the model and only 0.11% response not represented by the model. The model equation in terms of coded variables can be indicated as follows:

Growth = 0.538875 + (0.021 A) + (0.255875 B) + (0.05225 C) + (0.171375 D) – (0.0655 AB) – (0.01863 AC) + (0.0125 AD) – (0.03375 BC) + (0.06725 CD) + (0.019375 ABC) – (0.055 ABD) – (0.02325 BCD) + (0.022875 ABCD)

Where A is pH, B is inoculum size, C is temperature and D is light.

A two level factorial design (2LFD) has been chosen to pre-optimize of culture conditions as screening to reduce the range of factors affect the growth of *H. pluvialis* and the important factors will be optimized later on using response surface methodology. From the ANOVA analysis in Table 4, pH was insignificant effect to the growth only from pH 5 to pH 9. Nevertheless, pH less than 5 and more than 9 may has effect because the culture will become too acidic or too alkaline which can give effect to the growth. Differs from the result, Norihiko et. al (2001) reported that there was no cell growth in the culture with initial pH 5.23 and the growth rate slightly low for pH 6.14.

Inoculum size range was from 5% to 40% over total volume. Increment of inoculum size is directly proportional to the increment of cell density in the culture. Hence, 40% was found as the best inoculum size to maximize the culture growth.

In this study, optimum temperature for the optimum growth was found at 30°C. Previously, it was reported that optimum temperature for efficient biomass production of *H. pluvialis* in a range of 25°C and 30°C for astaxanthin production (Usha et. al., 2002; Norihiko, et. al., 2001). Dominguez-Bocanegra et. al. (2004) reported that maximum *H.pluvialis* growth was 3.5 x 10^5 cells/ml at 28°C. In contrast, Orosa et. al. (2005) used temperature 18°C to grow the culture. Various temperatures range from previous study, (15°C to 30°C) and 30°C was found as the optimum temperature for *H.pluvialis* growth as suggested by ANOVA analysis. Light quantity was more important than light intensity.

TABLE III
TWO LEVEL FACTORIAL DESIGN OF VARIABLES AND THE PREDICTED AND EXPERIMENTAL RESPONSE

Run	Variables				Response (Growth OD 680nm)	
	X1	X2	X3	X4	Experimental	Predicted
1	1	1	-1	1	0.798	0.7930
2	1	-1	-1	1	0.508	0.4928
3	-1	-1	1	1	0.553	0.5378
4	-1	1	-1	1	0.999	1.0143
5	-1	-1	-1	-1	0.087	0.0820
6	1	1	-1	-1	0.664	0.6690
7	-1	1	1	-1	0.617	0.6220
8	-1	-1	-1	1	0.058	0.0630
9	-1	-1	1	-1	0.088	0.1033
10	1	-1	-1	-1	0.135	0.1503
11	1	-1	1	1	0.719	0.7240
12	1	-1	1	-1	0.116	0.1110
13	-1	1	1	1	1.097	1.0920
14	1	1	1	1	0.950	0.9653
15	-1	1	-1	-1	0.644	0.6288
16	1	1	1	-1	0.589	0.5738

TABLE IV
ANOVA ANALYSIS OF VARIABLES AFFECTING GROWTH OF *H. pluvialis*

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	
Model	1.806907	13	0.138993	134.9117839	0.0074	significant
A-pH	0.007056	1	0.007056	6.848823101	0.1202	
B-Inoculum size	1.047552	1	1.047552	1016.794225	0.0010	
C-Temperature	0.043681	1	0.043681	42.39844698	0.0228	
D-Light	0.469910	1	0.469910	456.1128367	0.0022	
AB	0.068644	1	0.068644	66.62848823	0.0147	
AC	0.005550	1	0.005550	5.38728464	0.1460	
AD	0.00250	1	0.002500	2.426595487	0.2596	
BC	0.018225	1	0.018225	17.6898811	0.0521	
CD	0.072361	1	0.072361	70.2363504	0.0139	
ABC	0.006006	1	0.006006	5.829895656	0.1371	
ABD	0.04840	1	0.048400	46.97888862	0.0206	
BCD	0.008649	1	0.008649	8.395049745	0.1013	
ABCD	0.008372	1	0.008372	8.126425625	0.1042	
Residual	0.002061	2	0.001030			
Cor Total	1.808968	15				

$R^2 = 0.9989$, R = 0.9915, Std. Dev = 0.032

Continuous illumination was preferred over light/dark cycle for *H.pluvialis* growth (Kobayashi et. al, 1992; Fabregas et. al., 2001). Light supported the growth through photosynthesis process. Results from our experiments showed that presence of light is an important factor to optimize growth even acetate, was used as organic carbon source in the experiment.

To validate these predictions, the experiments perform by using the data shown in Table 5. The results showed that all experimental growth was higher than predicted. The most optimum growth was 1.178 which was 8.19% higher than predicted, 1.08879.

TABLE V
VALIDATION OF STATISTICAL MODEL BY APPLYING THE OPTIMIZED CONDITIONS

Run	pH	Inoculum size	Temperature	Light	Expected Growth	Experimental Growth
1	5.05	39.99	30.00	1.00	1.08879	1.178
2	5.47	39.97	30.00	1.00	1.08812	1.142
3	5.00	40.00	29.99	1.00	1.08097	1.129
4	5.00	40.00	23.81	1.00	1.07852	1.115
5	6.08	40.00	29.69	1.00	1.06606	1.093

IV. CONCLUSION

1. The pre-optimization of culture conditions on *H. pluvialis* growth was successfully achieved by using a two level factorial design (2LFD).

2. Inoculum size, temperature and light were found had significant effect ($P \leq 0.05$) whereas pH had insignificant effect ($P \geq 0.05$).

3. The optimize conditions for *H. pluvialis* growth are at pH 5, inoculum size of 40 %, temperature of 30°C and in the presence of light.

ACKNOWLEDGEMENT

The authors would like to thank Algaetech Sdn Bhd for providing stock culture of *Haematococcus pluvialis* under research collaboration with Kinetic and Fermentation System Design group, UKM. This research project was funded by Universiti Kebangsaan Malaysia under grant UKM-Industri-011-2011.

REFERENCES

[1] Dominguez-Bocanegra, A.R., Legaretta, I.G., Jeronimo, F.M. 2004. Influence of environmental and nutritional factors in the production of astaxanthin from Haematococcus pluvialis. Bioresource Technology 92: 209-214.

[2] Fabregas, J., Otero, A., Maseda, A., Dominguez, A. 2001. Two-stage cultures for the production of Astaxanthin from Haematococcus pluvialis. Journal of Biotechnology 89: 65-71.

[3] Gong, X., Chen, F . 1997. Influence of medium components on astxanthin content and production of Haematococcus pluvialis. Process Biochemistry 33(4): 385-391.

[4] Gu, W.L., An, G.H., Johnson, E.A. 1997. Ethanol Increases Caratenoid Production In Phaffia rhodozyma. Journal of Industrial Microbiology and Biotechnology 19:114 -117.

[5] Jeon, Y.C., Cho, C.W., Yun, Y.S. 2006. Combined effects of light intensity and acetate concentration on the growth of unicellular microalga Haematococcus pluvialis. Enzyme and Microbial Technology 39: 490-495.

[6] Johnson, E.A., Villa, T.G.. Lewis, M.J. 1980. Phaffia rhodozyma as an Astaxanthin Source in Salmonic Diets. Aqualculture 20: 123-134.

[7] Kang, C.D., Lee, J.S., Park, T.H., Sim, S.J. 2005. Comparison of heterotrophic and photoautotrophic induction on astaxanthin production by Haematococcus pluvialis. Applied Microbiology and Biotechnology 68: 237-241.

[8] Kobayashi, M., Kakizano, T., Nishio, N., Nagai, S. 1992. Effects of light intensity, light quality and illumination cycle on astaxanthin formation in a green alga, Haematococcus pluvialis. Journal ofFermentation and Bioengineering 74: 61-63.

[9] Kobayashi, M., Kakizano, T., Nagai, S. 1993. Enhanced caratenoid biosynthesis by oxidative stress in acetate-induced cyst cells of a green unicellular alga, Haematococcus pluvialis. Applied and Environmental Microbiology 59: 867-873.

[10] Kobayashi, M., Kurimura, Y., Tsuji, Y. 1997. Light-independent, astaxanthin production by the green microalga Haemtaococcus pluvialis under salt stress. Biotechnolology Letters 19: 507-509.

[11] Olaizola, M. 2000. Commersial production of astaxanthin from Haematococcus pluvialis using 25000-liter outdoor photobioreactors. Journal of Applied Phycology 12: 499- 506.

[12] Orosa, M., Franqueira, D., Cid, A., Abalde, J. 2005. Analysis and enhancement of astaxanthin accumulation in Haematoccus pluvialis. Bioresource Technology 96: 373-378.

[13] Najeeb Kaid Al-Shorgani, Aidil Abdul Hamid, Wan Mokhtar Wan Yusoff, Mohd Sahaid Kalil. 2013. Pre-optimization of medium for biobutanol production by a new isolate of solvent-producing Clostridium. BioResouces 8(1) : 1420- 1430.

[14] Norihiko, H., James, C.O., Yutaka, H., Hiroyuki, T., Hideo, T. 2001. Production of astaxanthin by Haematococcus pluvialis in a sequential heterotrophic-photoautotrophic culture. Journal of Applied Phycology 13: 395-402.

[15] Sarada, R., Sila, B., Ravishankar, G.A. 2002. Optimization of culture conditions for growth of the green alga Haematococcus pluvialis. World Journal of Microbiology and Biotechnology 18: 517- 521.

[16] Usha, T., Sarada, R., Ravishankar, G.A. 2002. Effect of culture conditions on growth of green alga- Haematococcus pluvialis and astaxanthin production. Acta Physiologiae Plantarul 24 (3) : 323-329.