OPTIMIZATION OF BATCH CULTIVATION OF *Chlorella* sp. BIOMASS USING RESPONSE SURFACE METHODOLOGY

Abstract

Optimal biomass production from microalgae using NPK medium; a relatively cheaper and locally available medium has been identified an important factor in the large-scale algae biomass production. In this study, various concentrations (0.3-0.7g/l) of NPK 20:20:20 were considered as source of nitrogen in the growth medium for *Chlorella* sp. Four independent parameters in algae culture (nitrogen concentration, pH, inoculum size and duration of the experiment at varying ranges were studied for maximum biomass and chlorophyll production. Response Surface Methodology (RSM) procedure result that nitrogen concentration and pH level are the dominant factors affecting biomass and chlorophyll production. Maximum biomass was achieved at 0.5 g/l N and 8.5 pH value. Higher N (0.8g/l) and lower N (0.3g/l) had minimal effect on biomass and chlorophyll production. There was a linear relationship between chlorophyll and biomass production while the residual nitrogen had an inverse relationship with the biomass production. Nitrogen concentration and pH were shown to be limiting factors under the conditions of the study. The inoculum size and duration of the experiment had minimal effect on biomass production.

Introduction

The global energy demand of the modern society and the climate change issues have stimulated researchers and policy makers to search for alternative and renewable energy that has little or no impact on the environment (Chisti, 2007, Al-lwayzy, *et al.,* 2014). The need to replace fossil fuel with renewable energy has been intensified since the beginning of the 20th century (Sutton, *et al*., 2014). Bioenergy is a sustainable source of greener and renewable energy, which has the potential to decrease dependency on fossil fuel without endangering the environment (Anitha & Narayanan, 2012). Among the bioenergy feedstock that has gained attention as a viable renewable source is the algal biomass.

Algal biomass can be manipulated to produce bio-ethanol, bio-diesel, biogas, bio-hydrogen fuel and bio-oil depending on the processing technology. Microalgae utilize various sources of carbon (organic or inorganic), to produce carbohydrates and lipids; that can be processed into biofuel (Rojan *et al*, 2011). They have the potential to yield more bio-oil (about 30 times more) than terrestrial oils seed crops (Sheehan *et al.,* 1998) without threat to food security or eco-system. Chisty, (2007), reported that algae biomass has the capacity of providing about 42% global biofuel, with some specie yielding as high as 94000liters of biofuel per hectare per annum. Thurmond, (2010) estimated the investment by both private and cooperate organization into algae culitivation and process to be over US$1 billion, due to the increased demand for utilization of algae biofuel in road, marine and aviation transportation to minimize greenhouse emissions.

Microalgae are a group of photosynthetic organisms that can be found in diverse environments including soil surface and most aquatic ecosystems (Nigam and Singh, 2011). They can also be categorized based on carbon supply source, while autotrophs utilize inorganic carbon in the presence of sunlight, heterotrophs use organic source of carbon. Some microalgae species (e.g. *Chlorella vulgaris)* can thrive in both

autotrophic and heterotrophic environment; hence are called mixotrophic algae (Liang *et al.*, 2009). They can be cultured in either open or closed pond system; in indoor or outdoor environment. To grow algae autotrophically, algae need nutrient, light and carbon source for the syntheses of all the biochemical compounds necessary for growth.

The biochemical components of algae biomass vary across species and culturing techniques. Some algae species has more lipids, carbohydrates and proteins production potential than others (Prabandono & Amin, 2015), therefore quality and quantity of biomass varies across species and production system (Vieira Costa & Morais, 2013). High biomass is produced in nutrient rich medium while lipids accumulates during nutrient starvation in algae culture. Various studies has reported high lipids and low biomass production in nutreint limiting algae culture (Zhu *et al*., 2016, Chen *et al*., 2018, Sharma *et al*., 2018, Chi *et al*., 2019). Benemann *et al*. (2018) observed that high light energy and low nitrogen can induce astaxanthin accumulation in *H. pluvialis* culture resulting in low biomass production. While Al-lwayzy *et al*.,(2014) argues that it is challenging to achieve maximum biomass and high lipid content at the same time in algae culture; reports of some studies have revealed that under some certain culture conditions, maximum biomass and high lipids content can be achieve. However, some of the imposed culture conditions can affect the biological activitites and cell composition in most algae species (Chi *et al*., 2019, Sajjadi *et al*., 2018).

Among the limiting parameters regulating biomass production are pH, temperature, nutrient, inoculum size, salinity etc. Although synthetic media are mainly used in algae cultivation, studies have shown that low cost growth media can be formulated with nitrate rich materials such as animal waste (Agwa, *et al.*,2012) and agricultural fertilizer (Whyte, 2018, AL-Mashhadani & Khudhair, 2017, Singh & Sikarwar, 2014,), sewage and industrial waste water (Pittman, *et al.,* 2011, Posadas *et al.*, 2017, Hupfauf *et al.*, 2016, Shchegolkova, *et al*., 2018) can also serve as a nutrient medium; especially for large scale algae cultivation. Using low cost and locally available materials for media formulation will reduce cost, preparation and the complexity of synthetic medium and subsequently the cost of biomass produced which is key for algae biofuel production.

The major component of algae nutrient medium is nitrogen (mainly in form of nitrate, nitrite, urea or Ammonia); required as an essential component of protein, phosphorus; an essential component of nucleic acid, carbon; required for energy, vitamins, trace elements. Each species of microalgae has its specific regulating and growth limiting parameters, though concentration, type and ratio for each nutrient type varies for different media. The optimal nutritional ratio for algal growth medium for N and P is 16: 1 (Sims *et al,* 2016). Stoichiometrically;

$$106CO_2 + 16NO_3 + HPO_4 + 18H \rightarrow C_{106}H_{263}O_{110}N_{16}P_1 + 138O_2. \quad \text{Eqn. (1)}$$

Optimization of growth parameters is vital in achieving maximum biomass production especially for sustainable biofuel production. For improvement in large scale biomass production from *Chlorella*, which is seen as a superior strain for biofuel production, it is vital that parameters such as pH, nitrate concntration,salinity and physical environment which has the tendency to alter the growth and the product yield significantly is optimised. In addition, interactions between the parameters and the effects on algal growth are also considered in the optimization process (Da Silva, *et al*., 2010).The optimal values of each parameter should support good algae growth and maximum biomass yield as well as been economically viable for commercial production. This process allows various variables to be considered at the same time by the use of statistical methods known as Response Surface Methodology (RSM).

RSM is used to identify the dependent and non- dependent variable in algae growth study. The first step in this experiment is the preliminary studies of various concentrations of nitrate in the growth medium and selection of ranges of concentrations among the experiment. The next step is the optimization procedure to determine the optimal concentration for maximum yield and their interaction with other variables. The Central composite design (CCD) is a tool in RSM that uses non-linear quadratic model to analyze variables. While RSM has been used in various algae studies to optimize and validate various media for lipid (Yang, *et al*., 2014), biomass, carbohydrates and lipid (Patel, *et al*., 2015, Panjak & Awasthi, 2015) and reactor processes (Da Silva,*et al.,* 2010), there are no local report on optimization of algal growth using NPK as nutrient medium.

The aim of this work was to use RSM techniques to optimize biomass and chlorophyll production in *Chlorella* sp using NPK 20:20:20 as a nutrient medium, and to identify key variables associated with quality biomass yield in algae cultivation.

MATERIALS AND METHODS

Algal strain isolation and culturing

Pond water containing micro algae was collected from (ARAC) African Regional Aquaculture Centre, Aluu, Rivers State and University of Port Harcourt Fish pond. Agar plating techniques were used to isolate algal strains from the raw Pond water. The algal strains were isolated by modifying the method of Anaga and Abu (1996). The isolated strains were sub-cultured in BG11 medium in natural illumination and subsequently sub culturing every seven days to maintain fresh algal culture. the isolates were purified by repeated streaking on solid media, and identified to the genus level using standard laboratory procedures and reference materials.

Scale Up

The NPK 20:20:20(Table1.) used were factory grade obtained from the farmers' market. Batch *Chlorella* sp culture was carried out in 250ml Erlenmeyer conical flask with 100ml working volume. The medium was autoclaved at 121^{o}C,15psi for 15min and all the culture parameters adjusted according to the designed experiment (Table 2). The culture was placed under solar irradiation and ambient temperature for the number of days of the experiment. The culture media were unaerated but manually agitated every 12 hours. Preculture inoculum with fixed biomass concentration with optical density 0.057 OD_{600} was added to the medium to start the experiment.

Analytical Procedure.

Biomass concentration was measured as optical density of the algae suspension with a spectrophotometer at 600nm wavelength (Spectronic 20, Genesys Thermos, USA). Total chlorophyll and residual nitrogen were measured by centrifuging 10mls of the growing culture at 5000rpm for 15min. The supernatant was decanted into an empty centrifuge tube and analyzed for the residual nitrogen by the Brucine method (Gopalan *et al*., 2008), while the wet paste at the bottom was washed 3x with distilled water and analyzed by the method of Burnison (1980) for total chlorophyll.

Table 1. Chemical composition of NPK 20:20:20

Components	**Concentration**
Total nitrogen Nitrate -nitrogen (N-$N0_3$) Ammonical-Nitrogen (N-NH_4) Urea -nitrogen (N-NH_2)	20% 6% 4% 10%
Phosphorus (P_2O5	20%
Potassium (K_2O	20%
Micro-nutrients	
EDTA-	Chelated
Iron (Fe)	1000ppm
Manganese (Mn)	500ppm
Boron (B)	200ppm
Zinc (Zn)	150ppm
Copper (Cu)	110ppm
Molybdenum (Mo)	70ppm

Response Surface Methodology. (RSM)

To obtain higher biomass yield, nitrogen concentration, pH, inoculum size and incubation time were considered as independent variables while biomass production, chlorophyll and residual nitrogen were dependent variables. The experiment was performed by Response Surface Methodology (RSM) using central composite design (Emeko *et al*., 2015) to determine the optimum levels of the significant variables and the effects of their mutual interactions on biomass production. Each independent variable was studied at three different levels (low, medium and high, coded as –1, 0 and +1, respectively) with the centre point of the design replicated three times for the estimation of error. Design Expert 10.0 software (Stat-Ease Inc. Minneapolis, USA) was used for experimental design, and data analysis. The central composite design and the values are shown in table 2. From this design, a second-order polynomial regression model (equation) was derived to define the response in terms of the independent variables.

$$\mathbf{Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_4 D + \beta_1 A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{44} D^2 + \beta_{12} AB + \beta_{13} AC + \beta_{14} AD + \beta_{23} BC + \beta_{24} BD + \beta_{34} CD} \quad \textbf{Eqn. (2)}$$

Where **Y**: Predicted response

A: pH, **B**: Nitrogen concentration, **C**: inoculum size **D**: Incubation time

β_0: Intercept, β_1, β_2, β_3 and β_4 are the linear coefficients, β_{11}, β_{22}, β_{33} and β_{44} are the squared coefficients, β_{13}, β_{14}, β_{23}, β_{24} and β_{34} are the interaction coefficients, A^2, B^2, C^2, D^2, **AB, AC, AD, BC, BD** and **CD** are the interactions between the variables as significant terms.

The independent variables are pH, nitrogen concentration, inoculum size and the duration of the experiment. The experimental Runs of 30 set were designed to achieve optimal growth parameters for algae growth (Table 2). The ranges of the minimum and maximum experimented variables are pH (7.5-9.5) Nitrogen concentration N (0.3-0.7g/l) Duration (6-14 days) and inoculum size (1.5-3.5%). Responses are biomass(mg/l), Total chlorophyll content (%) and Residual nitrogen (mg/l).

RESULT.

Response Surface Methodology

The response of the dependent variables analyzed with quadratic polynomial regression equation showed for biomass concentration, minimum and maximum output of 0.065(mg/L) and 1.265(mg/L) respectively, while mean and standard deviation were 0.6912 and 0.3936 respectively. Total chlorophyll showed minimum and maximum output of 0.008(%) and 0.182(%) respectively; mean and standard deviation of 0.0926 and 0.0568 respectively. The residual nitrogen showed minimum and maximum output of 0.021(mg/L) and 0.068(mg/L) respectively; mean and standard deviation of 0.0459 and 0.0148 respectively.

		Factor 1	**Factor 2**	**Factor 3**	**Factor 4**	**Response 1**	**Response 2**	**Response 3**
Std	Run	A: pH	B: [N] (g/L)	C: Duration (Day)	Inoculum Concentration (%)	Biomass (mg/L)	Chlorophyll (%)	Residual Nitrogen (mg/L)

25	1	8.5	0.5	10	2.5	1.205	0.161	0.024
30	2	8.5	0.5	10	2.5	1.215	0.165	0.026
16	3	9.0	0.6	12	3	0.527	0.102	0.066
19	4	8.5	0.3	10	2.5	0.065	0.008	0.054
29	5	8.5	0.5	10	2.5	1.236	0.182	0.027
3	6	8.0	0.6	8	2	0.782	0.101	0.047
14	7	9.0	0.4	12	3	0.119	0.015	0.061
20	8	8.5	0.7	10	2.5	0.479	0.077	0.037
6	9	9.0	0.4	12	2	0.121	0.029	0.048
9	10	8.0	0.4	8	3	0.847	0.103	0.045
22	11	8.5	0.5	14	2.5	1.102	0.113	0.021
10	12	9.0	0.4	8	3	0.147	0.019	0.054
27	13	8.5	0.5	10	2.5	1.174	0.168	0.038
23	14	8.5	0.5	10	1.5	1.167	0.163	0.032
21	15	8.5	0.5	6	2.5	0.875	0.122	0.041
2	16	9.0	0.4	8	2	0.148	0.024	0.063
26	17	8.5	0.5	10	2.5	1.265	0.169	0.021
5	18	8.0	0.4	12	2	0.592	0.053	0.065
1	19	8.0	0.4	8	2	0.632	0.041	0.044
24	20	8.5	0.5	10	3.5	0.963	0.128	0.041
18	21	9.5	0.5	10	2.5	0.392	0.032	0.068
7	22	8.0	0.6	12	2	0.882	0.126	0.042
11	23	8.0	0.6	8	3	0.775	0.097	0.041
4	24	9.0	0.6	8	2	0.379	0.042	0.048
8	25	9.0	0.6	12	2	0.334	0.025	0.059
17	26	7.5	0.5	10	2.5	0.342	0.028	0.066
13	27	8.0	0.4	12	3	0.752	0.125	0.055
28	28	8.5	0.5	10	2.5	1.219	0.177	0.023
12	29	9.0	0.6	8	3	0.463	0.077	0.058
15	30	8.0	0.6	12	3	0.538	0.105	0.061

Table 2. The optimization of the culture parameters of chlorella spp. using Central Composite Design

Analysis of Variance for Biomass Concentration, Total Chlorophyll and Residual nitrogen

ANOVA was used to justify the suitability of the model (Mei *et al.*, 2016). The model with high F-valve and low P-value (lower than 0.0500) indicates a significant model (Table 3). The ANOVA for the quadratic polynomial regression models demonstrated the significance of each model as indicated by the F- and P-values of responses .Biomass ($F\text{-}_{value}$6.89,$P\text{-}_{value}$0.0003) Chlorophyll ($F\text{-}_{value}$ 6.05, $P\text{-}_{value}$ 0.0007) and Residual nitrogen ($F\text{-}_{value}$3.67, $P\text{-}_{value}$0.0088). In the case of biomass concentration, the p-values indicate

the effect of linear effect coefficient of pH and nitrogen concentration, squared effect coefficient interactions of pH,nitrogen concentration and duration are significant model terms while other model terms are insignificant.

The model for Chlorophyll (Table 3) showed model F-value of 6.05 and P-value of 0.0007 is significant. There is only a 0.07% chance that an F-value this large could occur due to noise. The F and p-values suggest that the effect of pH and nitrogen concentration, quadratic interaction of pH,nitrogen concentration and duration improved chlorophyll production. The model also suggest that linear and quadratic interactions of duration and inoculum size have no effect on chlorophyll production from this study.

The model for residual nitrogen indicates significant P-value of 0.0088 and F-value of 3.67. there is only 0.88% chance that an F-value this large could occur due to noise. The p-values suggest the quadratic interactions of pH and nitrogen concentration on the residual nitrogen of the medium, while the linear interactions of pH, nitrogen concentration, duration and inoculum size are non- significant model terms. The lack of fit value of 3.47 implies that lack of fit is not significant relative to pure error. There is 9.10% chance this lack of fit F-value this large could occur due to noise.

The analysis of the interactive effect of pH,nitrate, duration and innoculum size were fitted with second order quadratic polynomial regression equations. The values of regression coefficient were calculated and fitted into the equation obtained for the prediction of optimized dependent variables as follows:

$$\text{Biomass}=1.22-0.1443*A+0.0896*B+0.0061*C-0.0046*D-0.2415*A^2-0.2652*B^2-0.0861*C^2-0.0670*D^2+08.0634*A*B+0.0148*A*C+0.0156*A*D+0.0045*B*C-0.0279*B*D-0.0177*C*D$$

Eqn.(3)

$$\text{Total Chlorophyll content}=0.1703-0.0171*A+0.0168*B+0.0024*C+0.0055*D-0.0378*A^2-0.0346*B^2—0.0159*C^2+0.0089D^2--0.0036A*C-0.0010A8D-0.0014B*C+0.0018*B*D+0.0016C*D$$

Eqn. (4)

$$\text{ResidualNitrate}=0.0265+0.0025*A+0.0020*B+0.0070*C+0.0018*D+0.0016*A^2+0.0062*B^2+0.0026*C^2+0039*D^2+0.0014*A*B-0.0022*A*C+0.0011*A*D+0.0007*B*C+0.0022*B*D=0.0021*C*D$$

Eqn. (5)

The coefficient estimates of the regression model for equations 3,4,5 as determined by ANOVA was presented in table 3. The second order polynomial regression equation was given in equation 2.

Table 3. The ANOVA result for Biomass, Chlorophyll and Residual nitrogen using a Quadratic Model

RESPONSE SURFACE PLOT.

Biomass concentration

The 3D response surface and 2D contour plot (Fig. 2a&b) depict the interactive effects of two variables and their individual effect on the biomass production (mg/L) in NPK medium. Fig.2a and 2b displayed effect of pH and nitrogen concentration on the biomass production. The optimal biomass concentration (1.265 mg/L) was achieved at the pH of 8.5 and nitrogen concentration of 0.5g/L, while the minimum biomass (0.065 mg/L) was produced at pH 8.5 and nitrogen concentration of 0.3 (g/L).This suggests the linear effect of pH (8.5) and nitrogen concentration (0.5g/L) were found to be dominant in optimized biomass production. The predicted biomass concentration was similar to the experimented values, thus showing the accuracy of the model (Table 4).

	BIOMASS			**CHLOROPHYLL**			**Residual Nitrogen**		
Terms	F-VALUE	P-VALUE	Coefficient estimates	F-VALUE	P-VALUE	Coefficient estimates	F-VALUE	P-VALUE	Coefficient estimates
Intercept			1.22			0.1703			0.0265
Model	6.89	0.0003	Significant	6.05	0.0007	Significant	3.67	0.0088	Significant
A-PH	12.39	0.0031	-0.1443	7.46	0.0155	-0.0171	1.61	0.2234	0.0025
B [N]	4.78	0.0451	0.0896	7.24	0.0168	0.0168	0.9578	0.3433	-0.0020
C-Duration	0.0220	0.8840	0.0061	0.1492	0.7047	0.0024	0.1253	0.7283	0.0007
D-Inoculum concentration	0.0125	0.9124	-0.0046	0.7728	0.3932	0.0055	0.8017	0.3847	0.0018
AB	1.59	0.2260	0.0634	0.1799	0.6775	0.0033	0.3441	0.5662	0.0014
AC	0.0864	0.7729	0.0148	0.2238	0.6430	-0.0036	0.7967	0.3862	-0.0022
AD	0.0969	0.7599	0.0156	0.0170	0.8976	-0.0010	0.1880	0.6708	0.0011
BC	0.0080	0.9297	0.0045	0.0024	0.9616	-0.0014	0.0787	0.7829	0.0007
BD	0.3084	0.5868	-0.0279	0.0522	0.8224	0.0018	0.7967	0.3862	0.0022
CD	0.1251	0.7285	-0.0177	0.0450	0.8349	0.0016	0.7083	0.4132	0.0021
A^2	39.67	<0.0001	-0.2415	41.66	<0.0001	-0.0378	38.09	<0.0001	0.0116
B2	47.86	<0.0001	-0.2652	35.05	<0.0001	-0.0346	10.89	0.0049	0.0062
C2	5.04	0.0402	-0.0861	7.38	0.0159	-0.0159	1.86	0.1928	0.0026
D2	3.05	0.1011	-0.0670	2.31	0.1493	0.0089	4.40	0.0532	0.0039
Residual									
Lack of Fit	64.63	0.0001	Significant	22.73	0.0015	Significant	3.47	0.0910	

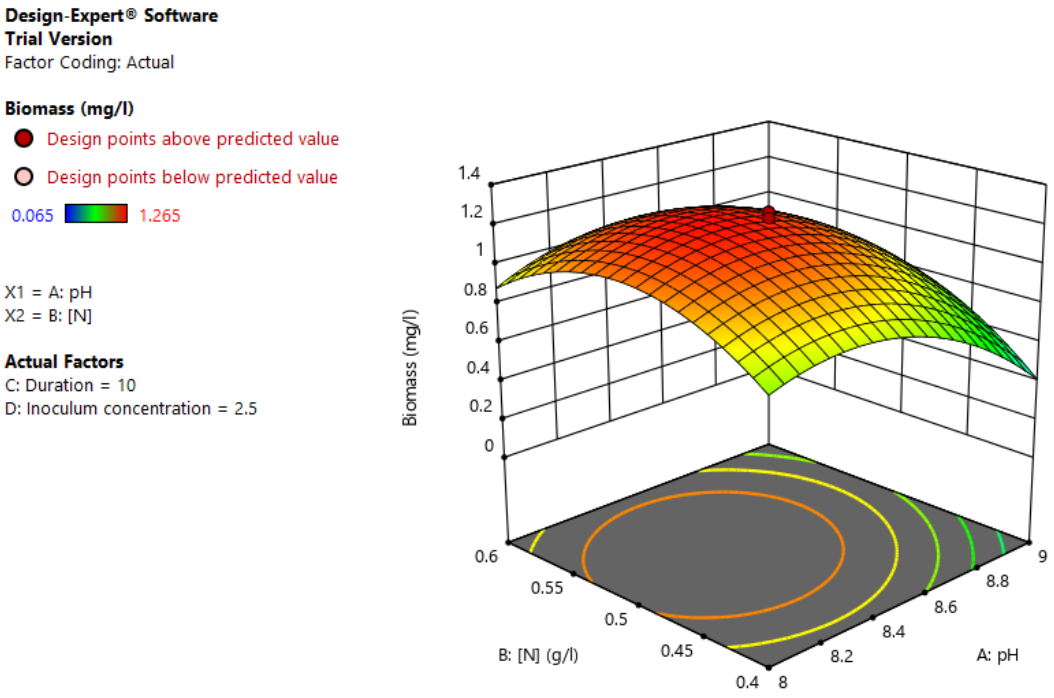

Figure 2.a.

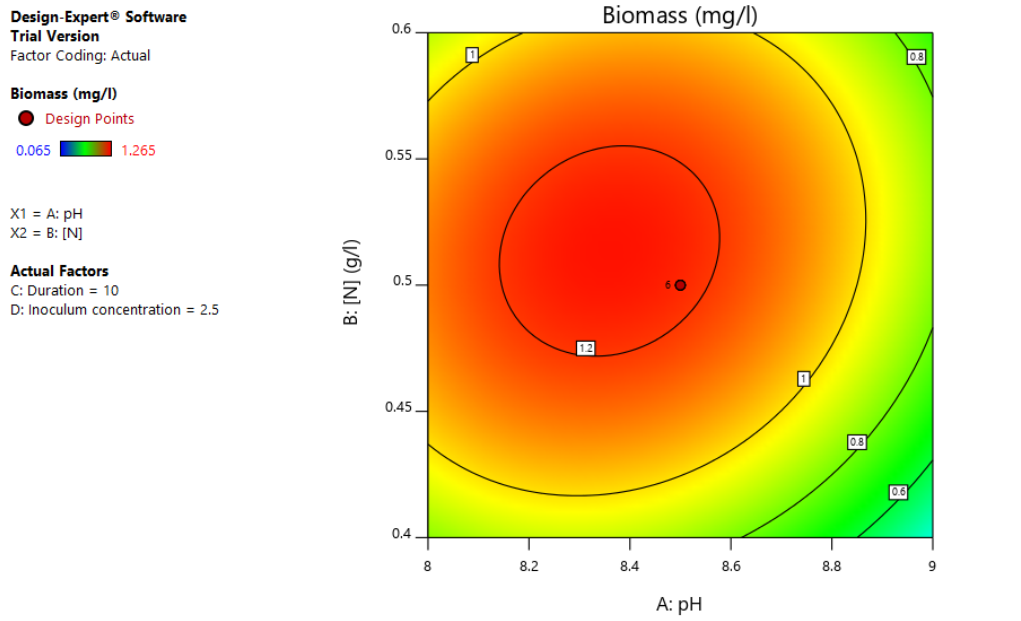

Figure 2.b.

Figures 2a&b. Surface 3D plot and 2D contour plot for Biomass concentration (mg/L**)** respectively.

Total Chlorophyll

Figures 3a and b showed that pH and nitrogen concentration affect the percentage of chlorophyll produced as suggested by the ANOVA P-value and F-value. The optimal chlorophyll of 0.168% was achieved with pH of 8.5 and nitrogen concentration of 0.5 g/L on day 10 and inoculum concentration of 2.5%, while the lowest chlorophyll (0.008%) was achieved at pH level of 8.5 and nitrogen concentration of 0.3(g/L). The percentage of actual chlorophyll content is within range predicted as shown in Table 4.

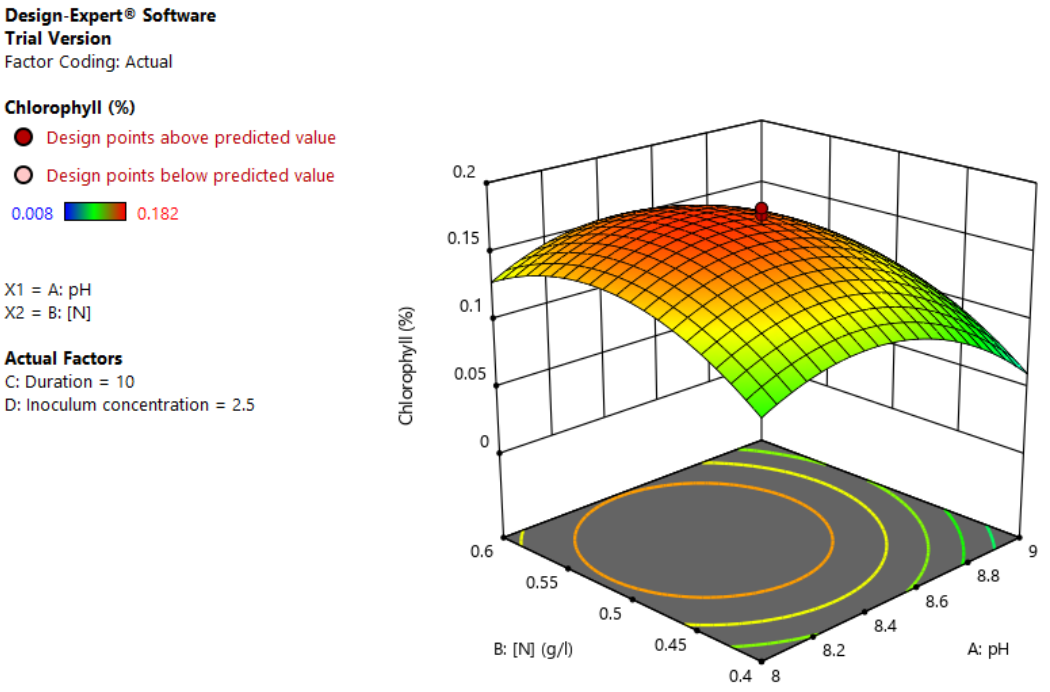

Figure 3.a.

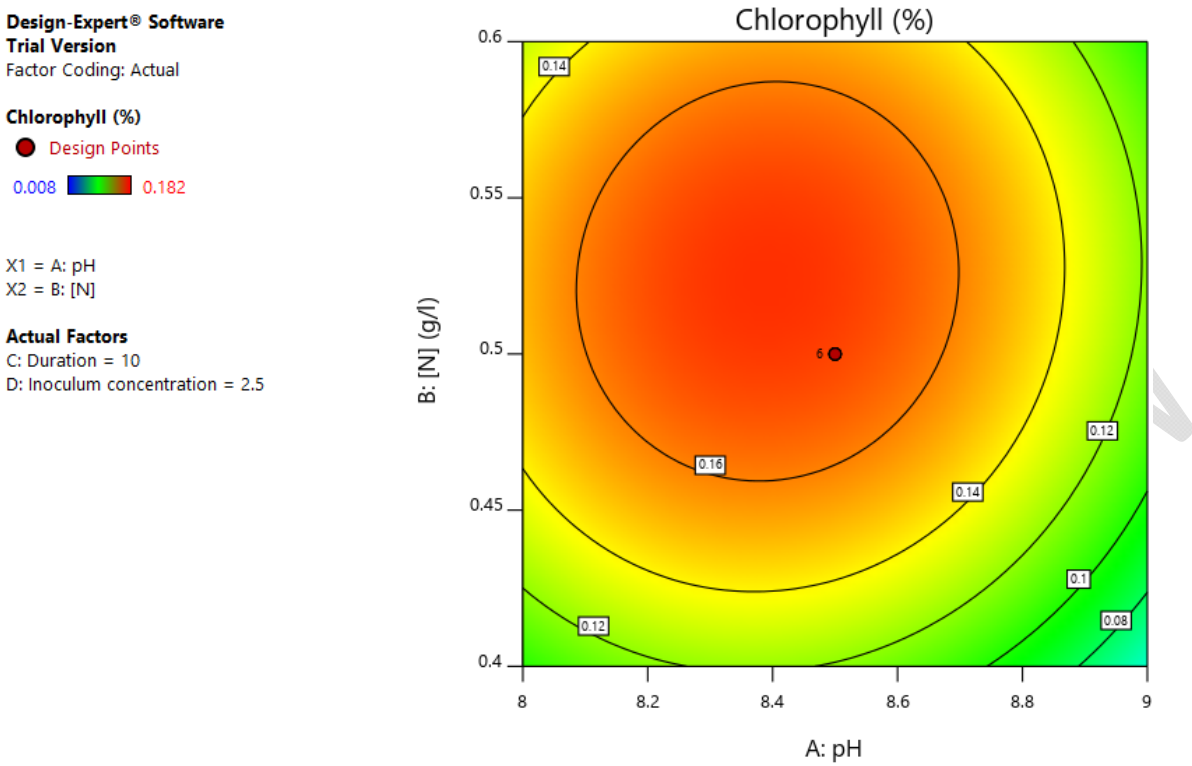

Figure 3.b.

Figures 3a&b. Surface 3D plot and 2D contour plot for Chlorophyll (%) respectively.

Residual Nitrogen

The 3D surface plot (Fig 4a) and Contour plot (Fig.4b) describe the effects of interactions of the independent variables on the value of the residual nitrogen. None of the independent variables (pH, nitrogen concentration, duration and inoculum size) has linear interactions with the residual nitrogen as displayed in the 3D surface plot (Fig.4a). The residual nitrogen values (0.021-0.068mg/L) vary with ranges of pH (8-9) and nitrogen concentration (0.4-0.6mg/L). The contour plot (Fig.4b), showed that the value of the residual nitrogen is at minimum between ranges of 0.45-0.58(mg/L) of nitrogen and 8.3-8.8 of pH value, with the lowest residual value at 8.5 pH value and 0.5(mg/L) of nitrogen concentration.

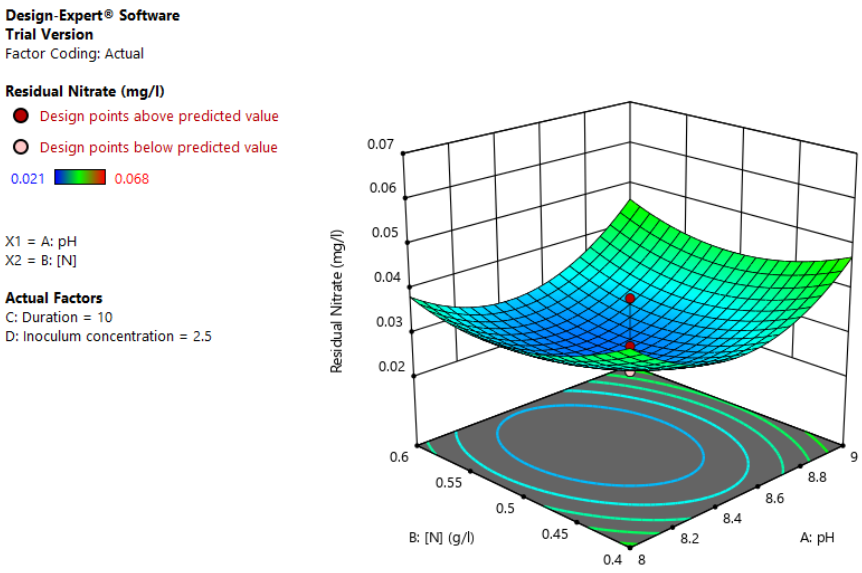

Figure 4.a.

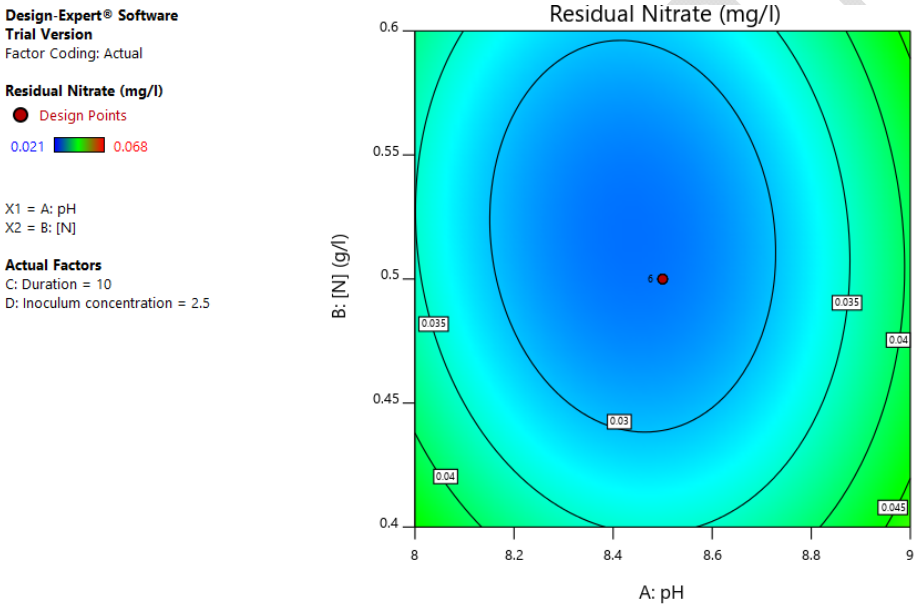

Figure 4. b.

Figures 4 a&b. Surface 3D plot and 2D contour plot for residual nitrogen (mg/L) respectively

Table 4. The Predicted and Actual values for Biomass, Chlorophyll and residual nitrogen as Determined by RSM

Run Order	Biomass		Chlorophyll		Residual Nitrogen	
	Actual Value	**Predicted Value**	**Actual Value**	**Predicted Value**	**Actual Value**	**Predicted Value**
1	1.21	1.22	0.1610	0.1703	0.0240	0.0265
2	1.22	1.22	0.1650	0.1703	0.0260	0.0265
3	0.5270	0.5588	0.1020	0.0797	0.0660	0.0590
4	0.0650	-0.0210	0.0080	-0.0019	0.0540	0.0551
5	1.24	1.22	0.1820	0.1703	0.0270	0.0265
6	0.7820	0.7643	0.1010	0.0943	0.0470	0.0403
7	0.1190	0.2996	0.0150	0.0423	0.0610	0.0543
8	0.4790	0.3373	0.0770	0.0654	0.0370	0.0473
9	0.1210	0.2572	0.0290	0.0265	0.0480	0.0489
10	0.8470	0.7159	0.1030	0.0776	0.0450	0.0458
11	1.10	0.8868	0.1130	0.1116	0.0210	0.0381
12	0.1470	0.3024	0.0190	0.0422	0.0540	0.0545
13	1.17	1.22	0.1680	0.1703	0.0380	0.0265
14	1.17	0.9603	0.1630	0.1238	0.0320	0.0386
15	0.8750	0.8625	0.1220	0.1019	0.0410	0.0353
16	0.1480	0.1891	0.0240	0.0329	0.0630	0.0573
17	1.26	1.22	0.1690	0.1703	0.0210	0.0265
18	0.5920	0.6742	0.0530	0.0724	0.0650	0.0532
19	0.6320	0.6651	0.0410	0.0643	0.0440	0.0529
20	0.9630	0.9420	0.1280	0.1458	0.0410	0.0458
21	0.3920	-0.0353	0.0320	-0.0149	0.0680	0.0778
22	0.8820	0.7914	0.1260	0.1038	0.0420	0.0434
23	0.7750	0.7036	0.0970	0.1005	0.0410	0.0420
24	0.3790	0.5418	0.0420	0.0758	0.0480	0.0505
25	0.3340	0.6279	0.0250	0.0709	0.0590	0.0448
26	0.3420	0.5417	0.0280	0.0534	0.0660	0.0676
27	0.7520	0.6541	0.1250	0.0922	0.0550	0.0544
28	1.22	1.22	0.1770	0.1703	0.0230	0.0265
29	0.4630	0.5436	0.0770	0.0781	0.0580	0.0565
30	0.5380	0.6597	0.1050	0.1166	0.0610	0.0533

Discussions

Biomass concentration

In this study, RSM was used to optimize biomass production from microalgae culture. The four independent factors (pH, Nitrate, duration and inoculum sizes) were varied to determine their individual and combine effect in improving biomass and chlorophyll production in a batch photoautotrophically cultured *Chlorella* sp using NPK 20:20:20 as nitrogen source. The maximum biomass yield (0.0287 μ/d) was achieved at 0.5g/L of nitrogen and pH value of 8.5. The minimum biomass (0.065mg/L) was produced at 0.3(g/L) nitrogen concentration. This result agrees with María de Lourdes *et al*.(2017) who report maximum biomass production of 0.205μ/d at N-NH_3 concentration of 0.49g/l in nitrogen and phosphrus removal in wastewater with *Chlorella vulgaris.* Ernst *et al.* (2005) also reported maximum cell density at 0.45g/l of nitrogen concentration with *Synechococcus* sp culture. Nitrogen in various form is known to be essential component in biomass biochemical, pigment (chlorophyll), amino -acid formation (Chen *et al*., 2009). High nitrogen concentration (about 0.8g/l) negatively affects algae growth while low concentration has no effect on algal biomass production (Chen *et al*.,2009, Gardner *et al*., 2011). Improved biomass production is also linked to the pH of the culture medium. Algae thrive well in alkaline environment; the optimum pH level for most algae species is between 7.5-9.5 with optimum pH range of 8.2-8.7value (Lavens & Sorgeloos, 1996, Kim *et al*., 2015, Eze *et al*., 2018). In this study, the initial culture pH value set of 8.5 achieved both maximum biomass concentration (1.265 mg/L) and minimum boimass cencetration(0.065) at different levels of nitrogen concentration. This suggests that nitrogen concentration or algal cell metabolism rather than carbon source governs the pH effect in biomass production .

Chlorophyll is a green pigment which determines the photosynthetic capacity and growth of algae. Maximun chlorophyll content (0.182%) was achieved at nitrogen concentration 0.5g/L and pH 8.5 on experiment day 10. The result from this study suggests that biomass concentration and chlorophyll content have a linear relationship, thus chlorophyll is found to be increasing with increase in biomass concentration (Da Silveira *et al.*, 2003, Soratto *et al.,* 2004)). Chlorophyll is an effective indicator of algae bloom in water bodies as a result of eutrophcation (Mencfel, 2013).

The result of this study showed evidence of inverse relationship between the biomass concentarion and residual nitrogen . The increase in biomass concentration results in decrease inResidual nitrogen . The lowest value for residual nitrogen (0.021mg/L) coincide with high value of biomass concentration (1,265 mg/L). This result agrees with various studies on nitrogen removal from waste water through algae biomass production.((Acevedo *et al*., 2017, Abdel-Raouf *et al*., 2012.). The rate of nitrogen removal and biomass production also depends on the concentration of nitrogen in the medium. Study by Acevedo *et al*. (2017) also found more algae biomass was produced at 40mg/L of N when compare to 90 and 150mg/L of N. This an indication of nutrient limitation or limiting conditions. These conditions are hyperbolic rather than linear relationship.

Conclusion.

The result of this study has demonstrated the use of RSM for the optimization of photoautotrophic batch culture of *Chlorella* sp in NPK medium to improve biomass production. The independent culture variables considered suggest that nitrogen concentration and the pH level are limiting factors in algal biomass production. The low biomass concentration at high inoculum size indicates that biomass production is not

influenced by inoculum size. The varying duration of experiment does not effect biomass concentraion, this is due to the fact that algae multiply daily. The optimal concentration of nitrogen established in this study suggets that large scale algae culture can be achieved with 0.5g/l of NPK, making it cheaper alternative to conventional synthetic medium, such as BG11.

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