Nature Environment and Pollution Technology
An International Quarterly Scientific Journal

p-ISSN: 0972-6268
(Print copies up to 2016)
e-ISSN: 2395-3454

Vol. 20 | No. 5 (Suppl) | pp. 2111-2116 | 2021

Original Research Paper | https://doi.org/10.46488/NEPT.2021.v20i05.028 | Open Access Journal

Optimization of *Chlorella* Culture Conditions with Response Surface Methodology to Increase Biomass

R. Kanimozhi*, D. Arvind Prasath*†, R. Dhandapani and Santhosh Sigamani****
*Medical Microbiology Laboratory, Department of Microbiology, School of Biosciences, Periyar University, Salem-636011, India
**Fermentation Technology Laboratory, Department of Microbiology, School of Biosciences, Periyar University, Salem-636011, India
†Corresponding author: D. Arvind Prasath; prasanthvi@periyaruniversity.ac.in

Nat. Env. & Poll. Tech.
Website: www.neptjournal.com

Received: 11-06-2021
Revised: 05-07-2021
Accepted: 12-07-2021

Key Words:
Response surface methodology
Biomass
optimization
Chlorella sp.

ABSTRACT

Microalgae is gaining popularity as a major ingredient in nutrition supplements. To mass cultivate, it is imperative to improve the biomass yield hence optimization of cultures conditions becomes paramount. In this work, an attempt has been made to optimize the microalgal production using response surface methodology (RSM) and validate further the optimized parameters. The optimum conditions for the cultivation of *Chlorella* sp. KPU016 under optimized nutrient conditions were pH 8.2, the light intensity of 3100 lx, glycerol 1.44 g.L^{-1} (under pre-set conditions of 12 h lighting, the temperature at 27±1°C. With these RSM-driven optimum conditions, the yield of microalgal biomass achieved was 282.50 mg.L^{-1}. For larger-scale microalgal harvesting, the validated optimal conditions can be inferred as the best for enhanced microalgal production. The isolate was partially sequenced and submitted to the NCBI database and the GenBank accession number is MZ348364.

INTRODUCTION

Cyanobacteria (CBs) are valuable mining sources to harness beneficial metabolites (proteins, fatty acids, carbohydrates, pigments, and antimicrobials), which can be augmented as feed-additives and health-care products. Among CBS, *Chlorella* is one among the most preferred for generating larger capacities of biomass yield owing to its high cell growth rate and the convenience to procure its biomass (Chinnasamy et al. 2009). Subsequently, they can be cultivated in an alkaline environment, thus eliminating or mitigating bacterial and fungal contamination (Ak 2011). However, the elevated cost impact in microalgae cultivation has hampered the large-scale harvesting of biomass for further commercial exploitation. The vast majority of the costs for larger-scale harvesting of microalgae cultivation are attributed to extensive consumption of media and water (Luo et al. 2016, Ortiz Montoya et al. 2014). Hence, using recycled water is deemed economical with promising potentials for harvesting biomass; simultaneously using by-products of other appliances as the substrate has added advantage, since this would decrease the cost and serve as a waste management step (Zamani et al. 2012). A variety of microalgae like *Chlorella vulgaris*, *Scendesmus obliques* and *Spirulina platensis* have been explored for cultivations in recycled water and a system with recycled nutrients (Ebrahimian et al. 2014). Amidst the explored microalgae, *Chlorella* has been acknowledged as the prominent one to be cultivated in different recycled waters, and it can sustain as well in simulated water with artificial media components (Chaiklahan et al. 2010). Many credible research works have established that the medium components impact the microalgal growth directly, thus the biomass output (Zhang et al. 2014). Meanwhile, the optimum growth conditions for mass cultivation of *Chlorella* were proven as pH 9.0 to10.0, temperature 25 to 30°C, light intensity (4000lx) (at a constant 12 h of light illumination under micro-aeration) (Daliry et al. 2017). However, these proven optimum conditions were under surplus and tailor-made nutrient conditions for *Chlorella* harvesting. It can be thus postulated that in a large-scale commercial platform for harnessing *Chlorella,* the parameters and factors involved has to be optimized. Regarding the *Chlorella* cultivated in recycled water, there are very limited credible works on the strategic enhancement of *Chlorella* biomass production, and of optimization of environmental conditions using response surface methodology (RSM) (Wang et al. 2021, Elyemni et al. 2021). Additionally, more research knowledge on the impact of recycled waste or organic carbon effects on *Chlorella* growth needs to be deeply investigated. Hence, in this study, *Chlorella* is cultivated in recycled water for biomass yield with glycerol (a by-product) as recycled-waste treatment. The optimum conditions for *Chlorella* cultivation were proven with the aid of Box-Behnken RSM, and the optimized conditions were further validated through an independent run. The interactive effects of pH, light intensity, and glycerol were explored based on biomass generation. *Chlorella* has also been strongly established over the years as a worthy source of nutraceuticals owing to their rich composition in terms of vitamins, antioxidants, etc., (Matos et al. 2019, Andrade et al. 2018). Hence this current work's proven optimum growth conditions can be further explored in a large-scale commercial application to procure biomass in higher volume so that it can be commercially exploited as a nutraceutical.

MATERIALS AND METHODS

Microalgal Strain, Media and Cultivation

The microalgal strain used in this study was isolated from Nangavalli lake, Salem, Tamilnadu, India (Latitude 11.7622ºN, Longitude 77.8898 ºE). Microalgal cells that were grown in BG 11 medium were used for the isolation of cyanobacteria. 1.0 mL of the cyanobacterial samples were transferred to sterile 100 mL of BG 11 medium in 250 mL conical flasks, in a growth chamber at 28±2ºC with the illumination of 2000lx with the aid of cool white 40 W fluorescent tubes (Philips). After 15 days of incubation, algae was harvested and used for further experiments (Yang et al. 2015). Glycerol was obtained as a waste residue from a biodiesel production facility and used in the optimization experiments.

Evaluation of Effective Parameters on Microalgae Growth

The RSM Box-Behnken design through Design Expert software (Version 11) was employed to investigate the effects of the parameters on the microalgal biomass yield; pH, Light intensity (lx), glycerol ($g.L^{-1}$) to predict the optimum conditions. The RSM is a combination of mathematical and statistical tools adequate for the analysis, modeling, and prediction of a particular response or multi-responses based on Design of Experiments (DOE) in which the interaction of several variables contributes to the outcome. In this work, the experimental runs were designed and performed involving the variables in ranges (pH, light intensity, glycerol load) as exhibited in Table 1. The original polynomial models based on the various parameters were fitted to experimental data

Table 1: RSM Box-Behnken design with the actual and predicted response.

	Factor 1	Factor 2	Factor 3	Response actual Vs Predicted	
Run	A: pH	B: Light intensity (lx)	C: Glycerol ($g.L^{-1}$)	Biomass ($mg.L^{-1}$)	Biomass ($mg.L^{-1}$)
1	9	3000	0.5	200.5	200.99
2	8	4000	2	266.6	264.11
3	8	3000	1.25	245.5	245.7
4	7	3000	0.5	198.2	197.41
5	8	3000	1.25	246.2	245.7
6	9	3000	2	254.2	254.99
7	8	4000	0.5	205.5	203.31
8	9	2000	1.25	233.6	230.62
9	9	4000	1.25	225.8	227.5
10	8	2000	2	268.4	270.59
11	7	4000	1.25	230.1	233.08
12	7	2000	1.25	238.9	237.2
13	8	3000	1.25	244.9	245.7
14	7	3000	2	271.2	270.71
15	8	3000	1.25	246.1	245.7
16	8	2000	0.5	201.6	204.09
17	8	3000	1.25	245.8	245.7

Model prediction R^2 for the quadratic polynomial equation is 0.9877 using the least-squares method and analysis of variance techniques in Design-Expert software (Version 11) (Zhai et al. 2017, Wang et al. 2021). The models for microalgal biomass yield, for the autotrophic cultivation of the microalga, could be obtained by Eq. (1)

$$Y=\beta 0 + \Sigma\beta iXi + \Sigma\beta ijXiXj + \Sigma\beta iiX2i, \quad \ldots(1)$$

Where Y is the predicted response, β_0 is the intercept term, β_i is the linear coefficient, β_{ij} is the quadratic coefficient, β_{ii} is the interaction coefficient, and X_i X_j represent the independent variables (Onumaegbu et al. 2019).

Analytical Methods

The *Chlorella* biomass was accumulated and measured once in 24 h by measuring the optical density at 560 nm. The dry biomass weight was measured by filtering 10 mL of each sample through a pre-weighed 0.45 nm membrane filter, drying in a drying chamber at 110℃ for 2 h, and reweighing the filter using an electronic-balance (Zhai et al. 2017).

RESULTS AND DISCUSSION

Determination of the Optimum Levels of the Factors

The range of pH, light intensity and glycerol, all impacted *Chlorella* biomass yield, significantly ($p < 0.01$) (Table 2). These three variables also positively impact the biomass yield individually (equation 1). The pH and daily illumination positively interacted with each other and also affected positively the response. PH and glycerol interacted negatively with each other, so did the light intensity, and glycerol pH^2, Light $intensity^2$, $Glycerol^2$ also interacted slightly negatively on the biomass yield even though pH x glycerol, pH^2, Light $intensity^2$, $Glycerol^2$ were significant at ($p < 0.01$) (Table 2) (Tork et al. 2017, Zhai et al. 2017).

Equations

Biomass = -478.837 + 149.054 x pH + 0.0258625 x Light intensity + 124.6 x Glycerol + 0.00025 x pH x Light intensity + -6.43333 x pH x Glycerol + -0.0019 x Light intensity x Glycerol + -9.05 x pH^2 + -4.55e-06 x Light $intensity^2$ + -10 x $Glycerol^2$ …(2)

In this current work, the optimum levels for the cultivation of *Chlorella* in recycled water with waste substrate glycerol were optimal, in contrast to other established results reported in previous studies. The pH, light intensity, and glycerol significantly impacted the *Chlorella* biomass output ($P < 0.01$). Previous research has also suggested that pH, light intensity, and carbon source play a significant influence in determining the outcome (Dorling et al., 1997, Fan et al. 2020, Zhai et al. 2017). When the pH value is more alkaline, the form of carbon shifts to form carbonates, hence the assimilation of glycerol is better suited at this pH range for *Chlorella*. Thus, the predicted optimum pH value between 8.2-8.6 is best suited for the cultivation of *Chlorella* in recycled wastewater under simulated conditions (Fig. 1a, 1b, & 1c). Regarding the light intensity, earlier published data has established that the microalgal composition, as well as the biomass, dwindle with respect to the extremes of light intensity (Pandey & Tiwari, 2010). Congruent results were achieved in our investigation. The *Chlorella* growth and biomass output were obtained at the optimum light inten-

Table 2: ANOVA of factors involved in the experiment.

Source	Sum of Squares	df	Mean Square	F-value	p-value
Model	8928.18	9	992.02	143.94	< 0.0001
A-pH	73.81	1	73.81	10.71	0.0136
B-Light intensity	26.28	1	26.28	3.81	0.0918
C-Glycerol	8102.64	1	8102.64	1175.70	< 0.0001
AB	0.2500	1	0.2500	0.0363	0.8544
AC	93.12	1	93.12	13.51	0.0079
BC	8.12	1	8.12	1.18	0.3136
A^2	344.85	1	344.85	50.04	0.0002
B^2	87.17	1	87.17	12.65	0.0093
C^2	133.22	1	133.22	19.33	0.0032
Residual	48.24	7	6.89		
Lack of Fit	47.14	3	15.71	57.14	0.0010
Pure Error	1.10	4	0.2750		
Cor Total	8976.42	16			

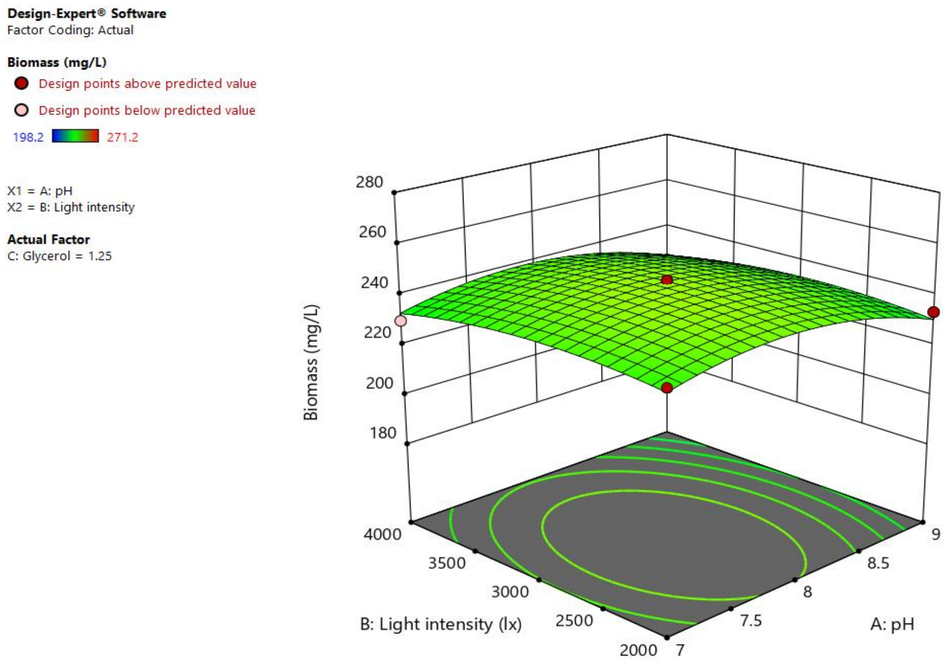

Fig. 1a: 3D contour plot of interaction between pH vs light intensity effect on biomass.

sity between 3100–3300lx in the current work; lesser than 4000lx as established in other published works. This result suggests that the current optimized level of light intensity could be economically viable via lesser energy consumption (Skorupskaite et al. 2015, Zhai et al. 2017).

The medium used for the cultivation of microalgae can be the prime factor when the culture conditions are to be optimized. In our work also glycerol levels were significant at concentrations 1.35 to 1.45 $g.L^{-1}$. Deep investigations have elucidated that the nutrients concentration and their forms

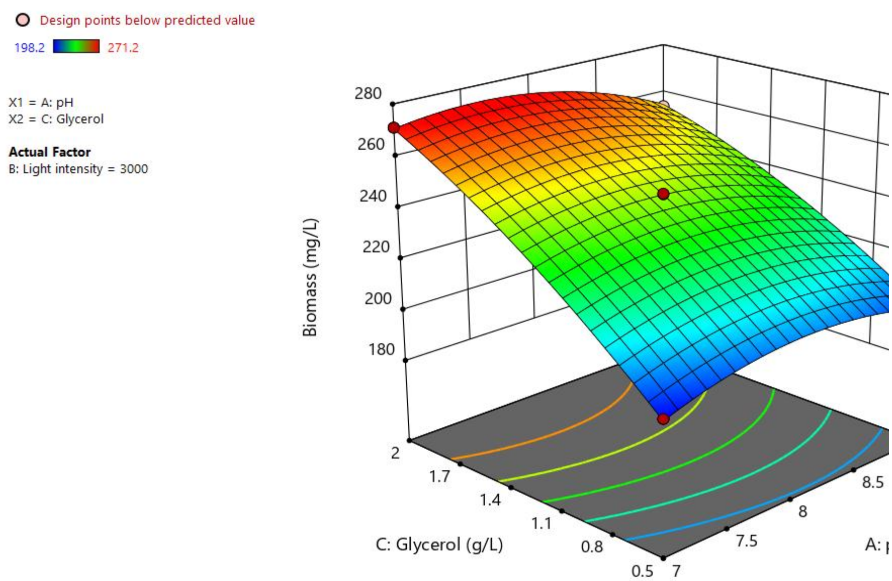

Fig. 1b: 3D contour plot of interaction between pH vs glycerol; their effect on biomass.

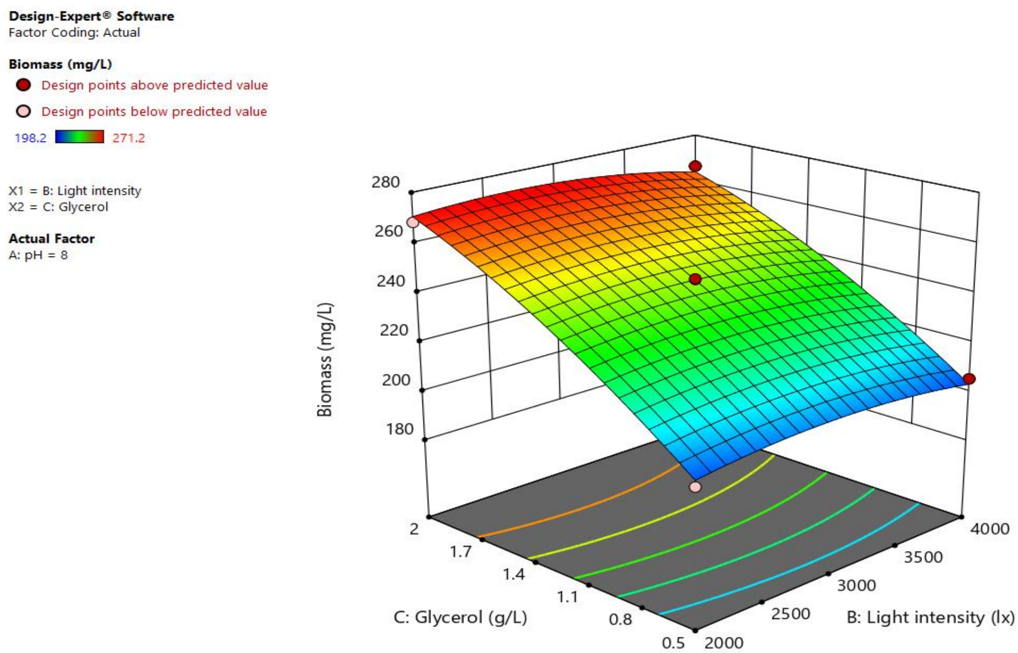

Fig. 1c: 3D contour plot of interaction between glycerol vs light intensity effect on biomass.

impacted the growth of *Chlorella* and other microalgae, and thus directly impacted *Chlorella* biomass generation and harvest.

Validated Run

The optimized values ascertained via RSM are pH 8.2, the light intensity of 3100 lx, glycerol 1.44 $g.L^{-1}$; when validated with an independent run, yielded 282.50 $mg.L^{-1}$ of *Chlorella* biomass.

CONCLUSION

In this work, the optimum levels of variables involved for the cultivation of *Chlorella* sp. KPU016 in simulated recycled water were envisioned and forecasted by RSM Box-Behnken and were further validated through an independent experimental run. All 17 runs projected minimal or no variation between the predicted values and experimental outcomes. Under the optimum conditions, pH 8.2, the light intensity of 3100 lx, glycerol 1.44 $g.L^{-1}$, when validated with an independent run, yielded 282.50 $mg.L^{-1}$ of *Chlorella* biomass. These optimal conditions could be strategies that can be a better endorsement for the advancement of *Chlorella* or other similar cyanobacteria cultivation so that the biomass can be harnessed in large-scale commercial applications. The isolate was partially sequenced and submitted to the NCBI database and the GenBank accession number was procured as MZ348364.1.

REFERENCES

Andrade, L.M., Andrade, C.J., Dias, M., Nascimento, C.A.O. and Mendes, M.A. 2018. Chlorella and spirulina microalgae as sources of functional foods, nutraceuticals, and food supplements; an overview. MOJ Food Process. Technol., 6(1): 45-58.

Ak, I. 2011. Effect of organic fertilizer on growth of blue-green alga *Spirulina platensis*. Aquacult. Int., 20: 413-422.

Chaiklahan, R., Chirasuwan, N., Siangdung, W., Paithoonrangsarid, K. and Bunnag, B. 2010. Cultivation of *Spirulina platensis* using pig wastewater in a semi-continuous process. J. Microbiol. Biotechnol., 20(3): 609-614.

Chinnasamy, S., Ramakrishnan, B., Bhatnagar, A. and Das, K.C. 2009. Biomass production potential of a wastewater alga *Chlorella vulgaris* ARC 1 under elevated levels of CO_2 and temperature. Int. J. Mol. Sci., 10(2): 518-532.

Daliry, S., Hallajisani, A., Roshandeh, J.M., Nouri, H. and Golzary, A. 2017. Investigation of optimal condition for *Chlorella vulgaris* microalgae growth. Global J. Environ. Sci. Manage., 3(2): 217-230.

Dorling, M., McAuley, P.J. and Hodge, H. 1997. Effect of pH on growth and carbon metabolism of maltose-releasing Chlorella (Chlorophyta). Eur. J. Phycol., 32(1): 19-24.

Ebrahimian, A., Kariminia, H.R. and Vosoughi, M. 2014. Lipid production in mixotrophic cultivation of *Chlorella vulgaris* in a mixture of primary and secondary municipal wastewater. Renew. Energy, 71: 502-508.

Elyemni, M., Louaste, B., Ouadrhiri, F.E., Bouia, A. and Eloutassi, N. 2021. Application of response surface methodology to optimize the extraction of essential oil from *Rosmarinus officinalis* using microwave-assisted hydrodistillation. J. Appl. Pharm. Sci., 11(01): 129-136.

Fan, H., Wang, K., Wang, C., Yu, F., He, X., MA, J. and Li, X. 2020. A comparative study on growth characters and nutrients removal from wastewater by two microalgae under optimized light regimes. Environ. Technol. Innov., 19: 100849.

Luo, L., He, H., Yang, C., Wen, S., Zeng, G., Wu, M., Zhou, Z. and Lou,

W. 2016. Nutrient removal and lipid production by *Coelastrella* sp. in anaerobically and aerobically treated swine wastewater. Bioresour. Technol., 216: 135-141.

Matos, J., Cardoso, C.L., Falé, P., Afonso, C.M. and Bandarra, N.M. 2019. Investigation of nutraceutical potential of the microalgae *Chlorella vulgaris* and *Arthrospira platensis*. Int. J. Food. Sci. Technol., 55: 303-312.

Onumaegbu, C., Alaswad, A., Rodriguez, C. and Olabi, A. 2019. Modeling and optimization of wet microalgae *Scenedesmus quadricauda* lipid extraction using microwave pre-treatment method and response surface methodology. Renew. Energy, 132: 1323-1331.

Ortiz Montoya, E.Y, Casazza, A.A, Aliakbarian, B., Perego, P., Converti, A. and de Carvalho, J.C. 2014. Production *of Chlorella vulgaris* as a source of essential fatty acids in a tubular photobioreactor continuously fed with air enriched with CO_2 at different concentrations. Biotechnol. Prog., 30(4): 916-22.

Pandey, J.P. and Tiwari, A. 2010. Optimization of biomass production of *Spirulina maxima*. J. Algal. Biomass Util., 1(2): 20-32.

Skorupskaite, V., Makarevicien, V. and Levisauskas, D. 2015. Optimization of mixotrophic cultivation of microalgae *Chlorella* sp. for biofuel production using response surface methodology. Algal Res., 2015: 7:45-50.

Tork, M.B., Khalilzadeh, R. and Kouchakzadeh, H. 2017. Efficient harvesting of marine *Chlorella vulgaris* microalgae utilizing cationic starch nanoparticles by response surface methodology. Bioresour. Technol., 243: 583-588.

Wang, Q., Oshita, K. and Takaoka, M. 2021. Evaluation of flocculation performance of amphoteric flocculant when harvesting microalgae *Coccomyxa* sp. KJ by response surface methodology. J. Environ. Manage., 277: 111449.

Yang, C.C., Wen, R.C., Shen, C.R. and Yao, D-J. 2015. Using a Microfluidic Gradient Generator to Characterize BG-11 Medium for the Growth of Cyanobacteria *Synechococcus elongatus* PCC7942. Micromachines, 6: 1755-1767.

Zamani, N., Noshadi, M., Amin, S., Niazi, A. and Ghasemi, Y. 2012. Effect of alginate structure and microalgae immobilization method on orthophosphate removal from wastewater. J. Appl. Phycol., 24(4): 649-656.

Zhai, J., Li, X., Li, W., Rahaman, H., Zhao, Y., Wei, B. and Wei, H. 2017. Optimization of biomass production and nutrients removal by *Spirulina platensis* from municipal wastewater. Ecol. Eng., 108: 83-92.

Zhang, C., Zhang, Y., Zhuang, B. and Zhou, X. 2014. Strategic enhancement of algal biomass, nutrient uptake, and lipid through statistical optimization of nutrient supplementation in coupling *Scenedesmus obliquus*-like microalgae cultivation and municipal wastewater treatment. Bioresour Technol., 171: 71-79.