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

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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 microalgal cultivation has hampered the large-scale harvesting of biomass for further commercial exploitation. The vast majority of the costs for larger-scale harvesting of microalgal cultivation are attributed to excessive 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, Scenedesmus 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 \textit{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. \textit{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

| Run | Factor 1 | Factor 2 | Factor 3 | Response actual Vs Predicted |
|-----|----------|----------|----------|-----------------------------|
| 1   | 9        | 3000     | 0.5      | 200.5                       |
| 2   | 8        | 4000     | 2        | 266.6                       |
| 3   | 8        | 3000     | 1.25     | 245.5                       |
| 4   | 7        | 3000     | 0.5      | 198.2                       |
| 5   | 8        | 3000     | 1.25     | 246.2                       |
| 6   | 9        | 3000     | 2        | 254.2                       |
| 7   | 8        | 4000     | 0.5      | 205.5                       |
| 8   | 9        | 2000     | 1.25     | 233.6                       |
| 9   | 9        | 4000     | 1.25     | 225.8                       |
| 10  | 8        | 2000     | 2        | 268.4                       |
| 11  | 7        | 4000     | 1.25     | 230.1                       |
| 12  | 7        | 2000     | 1.25     | 238.9                       |
| 13  | 8        | 3000     | 1.25     | 244.9                       |
| 14  | 7        | 3000     | 2        | 271.2                       |
| 15  | 8        | 3000     | 1.25     | 246.1                       |
| 16  | 8        | 2000     | 0.5      | 201.6                       |
| 17  | 8        | 3000     | 1.25     | 245.8                       |

Model prediction $R^2$ for the quadratic polynomial equation is 0.9877

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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)

\[ Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ij} X_i X_j + \sum \beta_{ii} X_i^2, \quad \ldots \quad (1) \]

Where \( Y \) is the predicted response, \( \beta_0 \) is the intercept term, \( \beta_i \) is the linear coefficient, \( \beta_{ij} \) is the quadratic coefficient, \( \beta_{ii} \) is the interaction coefficient, and \( X, X \) 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°C 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 pH², Light intensity², Glycerol² also interacted slightly negatively on the biomass yield even though pH x glycerol, pH², Light intensity², Glycerol² 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 pH² + -4.55e-06 x Light intensity² + -10 x Glycerol² \quad \ldots \quad (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-

| 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  |
| A²       | 344.85         | 1  | 344.85      | 50.04   | 0.0002  |
| B²       | 87.17          | 1  | 87.17       | 12.65   | 0.0093  |
| C²       | 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 |             |         |         |
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...
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.

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