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Optimization of the parameters of culture medium for chemo-photosynthetic carbon dioxide sequestration using response surface methodology

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Abstract

Culture conditions for maximum dry weight production were optimized using response surface methodology. Central Composite Design (CCD) was used to access the effect of three parameters i.e. HCO_3^- concentration, pH of culture medium and cobalt ion concentration on maximum dry weight production of green microalgae *Chlorella sorokiniana* PAZ. The experiments estimated the optimized conditions as, 1127 mg/l for HCO_3^-, 7.86 for pH and 13.07 mg/l for Co^{2+}. Highest predicted biomass for *Chlorella sorokiniana* PAZ was 0.891g/l using response surface methodology.

Keywords: Bicarbonates, Calcium, CO_2 sequestration**,** Response Surface Methodology

Introduction

Microalgae are the source of attraction for the number reasons like biofuel production carbon dioxide sequestration, pigment production, heavy-metal removal, sunscreen formulation, etc. (Chisti 2007; Ramanan *et al.,* 2010; Min *et al.,* 2011; Borowitzka 2013; Chi *et al.,* 2013). Microorganism isolated from unique habitat has its own mechanism in tolerating extreme conditions, which is necessary for its survival. Microalgae isolated from alkaline conditions possess an ability to grow at high pH and high ion strength. These properties provide an affordable and favourable approach in the selection of a carbon dioxide mitigation process (Chi *et al.,* 2011).

Most of CO_2 capture techniques are not environment friendly as they cause leakage of CO_2 back to the environment. Also, dissolution of CO_2 gas in water is 1.45 g/l at 25°C, which is very less most of flushed CO_2 into the water escapes and contaminate the atmosphere (Devgoswami *et al.,* 2011). Hence, use of safe method for CO_2 disposal is important. Trapping of CO_2 into the alkaline solution is a safer and environment-friendly approach. Calcium hydroxide (lime) is one of such alkaline solutions, which makes this process not only safe but also profitable due to its availability as waste from paper and pulp industry. CO_2 can readily be dissolved into the sedimentary form in lime solution. This aqueous form of CO_2 called as bicarbonate (HCO_3^-) is the preferred source of carbon for microalgae as they can import it into the cells through the cell membranes (Price *et al.,* 2008). Inside the cell near the site of Rubisco the bicarbonate is converted back to the CO_2. Enzyme carbonic anhydrase plays an important role in maintaining equilibrium between CO_2 and HCO_3^-.

Along with macronutrient such as carbon microalgae also requires heavy metals in trace quantities. Heavy metals are the part of an active site of many essential enzymes such as carbonic anhydrase. Among them zinc is essential for the functioning of carbonic anhydrase. It is believed that cobalt replaces the zinc and restores the activity of carbonic anhydrase in zinc limited cells of *Thalassiosira weissflogii* (Morel *et al.,* 1994). Tripathy *et al.* (1983) demonstrated that the presence of cobalt into the growth medium enhances the ratio of PS II to PS I.

Optimization of culture conditions is the necessity to achieve the maximum growth for an industrially important organisms (Mandal and Mallick 2009; Chi *et al.,* 2013; Kim *et al.,* 2013). The media optimization based on the Response Surface Methodology (RSM) gives the optimized values in a limited number of trial runs. Furthermore, process gives an idea about the interactions between the different factors used in optimization process and their effect on final response, which is sometimes impossible in the traditional experimental process. In the present study, an attempt is made to determine optimum conditions for the production of higher biomass using various factors such as the pH and concentrations of bicarbonate and cobalt in the medium. Optimized values for all three components were determined by Central Composite Design (CCD) and model is verified using statistical analysis.

Material and Methods

Isolation and maintenance of Chlorella sorokiniana PAZ

Microalgal culture was isolated from the Lonar lake situated in the Buldhana district of Maharashtra, India and was cultivated in BG-11 medium, incubated at 25±1°C and 40 µmole $m^{-2}s^{-1}$ with a 16 h light: 8 h dark photoperiod until visible growth observed. Solidified agar media was used for isolation and purification of the culture. The purity of culture was checked by repeated sub culturing and daily observation under the microscope (Nikon Eclipse 200).

Determination of growth rate

Growth rate was determined by inoculating culture series of flasks each containing 40 ml growth medium incubated at 25±1°C temperature and 40 µmole $m^{-2}s^{-1}$ (photon flux density) under 16 h : 8 h light/dark photoperiod. One flask was harvested at an interval of 24 h analysed for optical density at 750 nm, pH of culture and dry weight of biomass. The contents of the flask were centrifuged at 5000 × for 10 min. The pellet of microalgal biomass was dried in oven at 60°C for 24 h for constant weight. The samplings were done in triplicates.

CO_2 trapping and consumption

Trapping of gaseous CO_2 into aqueous phase was done in water containing varying concentrations of calcium oxide (50, 100, 150, 200 mg Ca/l). Undissolved matter from the medium was removed by filtration using Whatman's No.1 filter paper. In trapping experiments compressed CO_2 gas (99.9% purity) was used as the source of CO_2 at constant flow rate until the pH of a medium dropped to 6±0.1. Flasks were inoculated with *Chlorella sorokiniana* PAZ.

Tolerance to cobalt concentration

To check the tolerance of culture to cobalt a stock solution of cobalt (500 mg/l) was used to prepare final concentrations as 5, 10, 15 and 20 mg/l in BG-11 medium. Culture flask was inoculated with *Chlorella sorokiniana* PAZ and incubated at 25±1°C with 40 µmole $m^{-2}s^{-1}$ light intensity and 16:8 h as light to dark photoperiod.

Central Composite Design (CCD)

Central composite design was performed to determine the optimum culture conditions for growth of *Chlorella sorokiniana* PAZ using three different factors. Concentration of bicarbonate, cobalt and pH values was decided on the basis of previous experiment (data are not shown), and their coding level is as shown in Table 1. Experimental design consists of 20 runs in one block. Different concentrations of bicarbonates were prepared in the medium using $Ca(OH)_2$ water, to that exact amount of cobalt solution was added from the stock to make preferred cobalt concentration and pH was adjusted using 1M NaOH at a desired point. All the flasks were kept in uniform incubation conditions as 25±1°C temperature and 40 µmole $m^{-2}s^{-1}$ (photon flux density) with a 16 h light: 8 h dark photoperiod. After 10 days the biomass was filtered, washed twice with distilled water to remove all contaminates and dried overnight at 60°C. The experiments were carried out in replicate of three. Selected variable and response in terms of dry weight are given in Table 2.

For three factors, statistical design is defined by Eq. (1)

$$Y= \beta_0 + \beta_1 HCO_3^- + \beta_2 pH + \beta_3 Co^{2+} + \beta_{11} (HCO_3^-)^2 + \beta_{22} (pH)^2 + \beta_{33} (Co^{2+})^2 + \beta_{12} (HCO_3^-) (pH) + \beta_{13} (HCO_3^-)(Co^{2+}) + \beta_{23} (pH) (Co^{2+}) \quad \text{Eq. (1)}$$

Where, Y is the predicted response Dry weight (g/l), β_0, β_1, β_2, β_3, β_{11}, β_{22}, β_{33}, β_{12}, β_{13} and β_{23} are the coefficient of equation, and HCO_3^-, pH and Co^{2+} are the coded levels of variables. To predict the optimal condition for the production of higher biomass using carbon dioxide sequestration multiple regression analysis and ANOVA were performed using Minitab® 16.2.2 software.

Table 1 Coding levels for Central Composite Design (CCD) experiment

Coded levels	+1.68	-1.68	1	-1	0
HCO_3^- (mg/l)	1127	473	1000	600	800
pH	8.86	7.18	8.5	7.5	8
Co^{2+} (mg/l)	13.27	6.73	12	8	10

Table 2 Experiment design and results for CCD experiment

HCO_3^-	pH	Co^{2+}	Dry weight (g/l) Actual	Dry weight (g/l) Predicted
0.00	1.68	0.00	0.431	0.459
0.00	-1.68	0.00	0.495	0.454
1.00	-1.00	-1.00	0.615	0.617
1.00	1.00	-1.00	0.590	0.594
-1.00	-1.00	-1.00	0.349	0.358
0.00	0.00	0.00	0.702	0.707
0.00	0.00	0.00	0.697	0.707
1.00	-1.00	1.00	0.722	0.779
0.00	0.00	-1.68	0.501	0.526
0.00	0.00	0.00	0.705	0.707
-1.00	1.00	-1.00	0.495	0.445
-1.68	0.00	0.00	0.453	0.477
0.00	0.00	1.68	0.785	0.750
0.00	0.00	0.00	0.700	0.707
0.00	0.00	0.00	0.716	0.707
0.00	0.00	0.00	0.715	0.707
-1.00	-1.00	1.00	0.497	0.500
1.68	0.00	0.00	0.861	0.827
-1.00	1.00	1.00	0.554	0.558
1.00	1.00	1.00	0.728	0.726

Results and Discussion

Lonar Lake is formed due to meteoritic impact and has very high pH around 10. After adoption of the number of isolation techniques, various cyanobacterial and microalgal strains were isolated. *Chlorella sorokiniana* PAZ, one of the potential culture among others was isolated and deposited at National Repository for Cyanobacteria and Microgreen algae (Freshwater), DBT-ISBD, Imphal; Manipur with accession number BTA9030 (Fig. 1).

In the process of carbon dioxide fixation pH of a medium has an influencing effect on growth of culture and availability of inorganic carbon in the medium. Variation in pH can affect algal growth in a number of ways. It can affect the distribution of carbon dioxide species and carbon availability; alter the availability of trace elements and essential nutrients. A number of studies demonstrated that pH also changes significantly in the marine system despite the strong buffering capacity of carbonate systems (Jansson and Northen 2010). pH levels in marine systems appeared to correlate with changes in temperature, dissolved oxygen and algal growth. The comparative concentration of CO_2, HCO_3^-, and CO_3^{2-} of the carbonate system and pH is closely related to one other (Chenl and Durbin 1994). As pH increases, carbonate increases and bicarbonate and molecular CO_2 decreases. Microalga generally utilizes dissolved CO_2 in the form of bicarbonates, which are available abundantly at pH 8, and hence pH 8 was chosen for further studies.

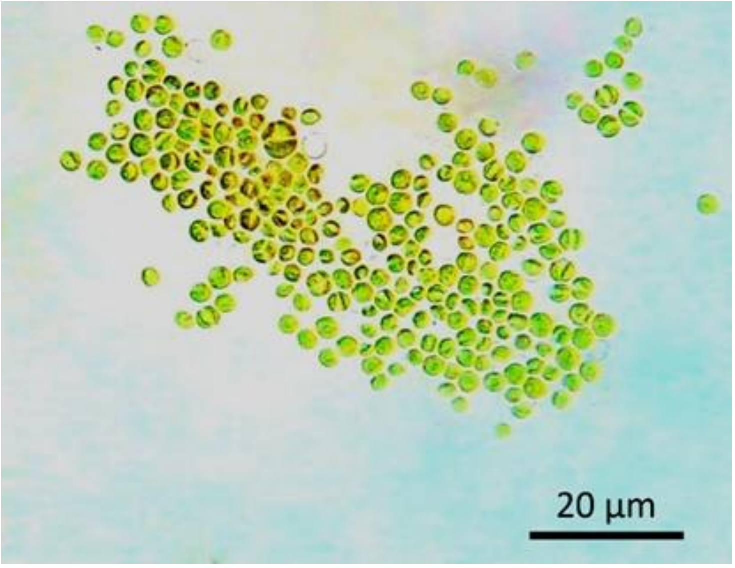

Fig. 1 *Chlorella sorokiniana* PAZ under 100× magnification

One of the limitations of carbon dioxide trapping in the soluble form is the low CO_2 dissolution rate and retention time in aqueous state (Devgoswami *et al.,* 2011). To avoid the problems associated with low dissolution rate of CO_2 a chemical scrubber should be made, which can allow maximum CO_2 to get dissolved in the medium and then can readily be utilized by microalgae for their growth. In order to resolve the problem, calcium hydroxide (CaOH) is used as the chemical scrubber (Velts *et al.,* 2011). Calcium hydroxide is readily available as waste from pulp and paper industry.

In the present study, CO_2 was trapped into CaOH water containing different concentrations of Ca as 50, 100, 150 and 200 mg/l. Fig. 2 shows the amount of bicarbonate formed and consumed by *Chlorella sorokiniana* PAZ. Although bicarbonates formed were maximum in medium containing 200 mg/l Ca but its utilization was to a smaller extent by *Chlorella sorokiniana* PAZ. Probable reason for the less utilization could be the inability of culture to grow at higher concentrations of calcium. Although more bicarbonates were formed, however, culture could not survive at increasing concentrations of calcium and tends to show declined growth and hence less utilization. From the experimental result, it can be noticed that the maximum utilization of bicarbonates and higher growth was achieved at 150 mg/l of Ca.

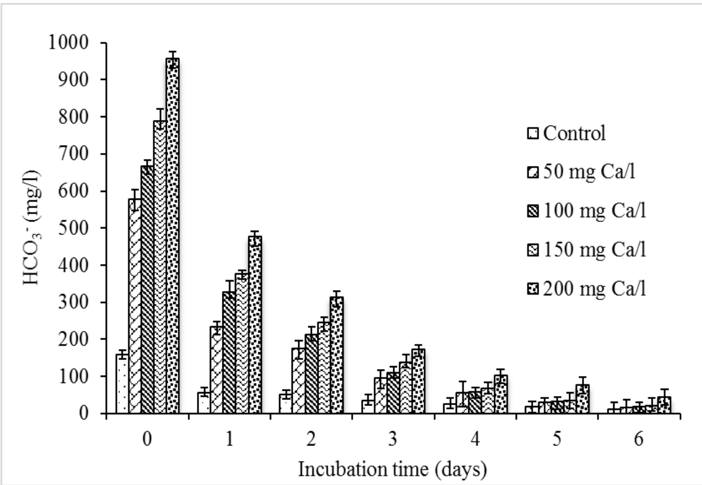

Fig. 2 Bicarbonate formation and consumption by *Chlorella sorokiniana* PAZ

Czerpak *et al.* (1994) showed that Co^{2+} in medium shows stimulatory effect on *Chlorella pyrenoidosa* with an overall increase in fresh weight, dry weight, chlorophylls a and b, total carotenoids, water-soluble proteins and monosaccharide content. Carbonic anhydrase (CA) is the most important enzyme in the carbon dioxide fixation that requires zinc as a cofactor. It has been found that cobalt replaces the zinc requirement in marine diatoms (Yee and Morel 1996). Tripathy *et al.* (1983) shown that cobalt restores the oxygenase but not the carboxylase function of ribulose bisphosphate carboxylase. Fig. 3a-b represents the chlorophyll a (mg/l) and dry weight (g/l) of *Chlorella sorokiniana* PAZ under different concentrations of cobalt into the medium. It has been found that cobalt concentration increases not only chlorophyll a but also dry weight of *Chlorella sorokiniana* PAZ up to 10 mg/l. However, substantial decrease in both the parameters was found at higher concentrations. These results are in agreement with previous reports (Lustigman *et al.*, 1995 and El-Sheekh *et al.*, 2003). Therefore, 10 mg/l cobalt concentration was selected for further optimization process.

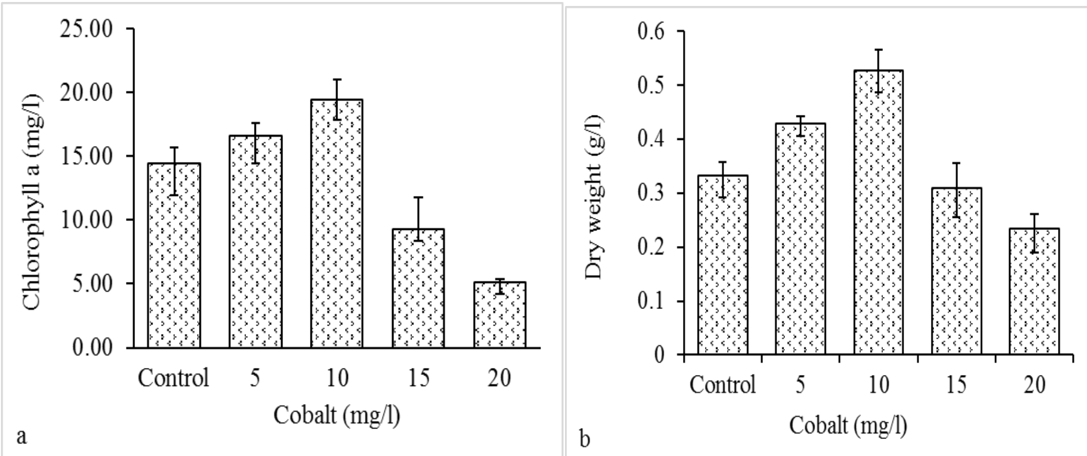

Fig. 3 Tolerance of *Chlorella sorokiniana* PAZ to different concentrations of cobalt (a) Chlorophyll *a*, mg/l (b) Cell dry weight, g/l

Response surface methodology was used to determine the conditions to produce high biomass through carbon dioxide sequestration. Jacob-Lopes *et al.* (2008) used RSM to determine the effect of temperature, light intensity and CO_2 concentration on rate of CO_2 removal using photobioreactor and concluded high light intensity, CO_2 at highest level and fixing temperature at lower level elevates the CO_2 removal from liquid phase. In a similar manner Kim *et al.* (2012) used RSM to optimize the culture conditions on mass production of three microalgae using three factors i.e. initial pH, nitrogen and phosphate concentration.

Table 3 shows the regression coefficient, F test and P test. Highest dry weight was observed for run 18, whereas the lowest value of dry weight was observed at run 5. Coefficient of all variables was calculated from Table 3 and used to present the Eq. (2)

$$Y = -26.1322 + 0.0035\ HCO_3^- + 5.9867\ pH + 0.2113\ Co^{2+} - 0.00\ (HCO_3^-)^2 - 0.3547\ (pH)^2 - 0.0064\ (Co^{2+})^2 - 0.0003\ (HCO_3^-)(pH) + 0.0000\ (HCO_3^-)(Co^{2+}) - 0.0073\ (pH)(Co^{2+})$$

Eq. (2)

Where, Y is predicted response, i.e. dry weight (g/l), HCO_3^- (mg/l), pH of the medium and concentration of Co^{2+} (mg/l) are the coded variables.

Table 3 Estimated regression coefficient, F test and P test (R^2 = 0.9655)

Term	Coefficient	F test	P test
Constant	-26.1322	**-9.565**	0.000
HCO_3^-	**0.0035**	3.124	**0.011**
pH	5.9867	**9.808**	**0.000**
Co^{2+}	**0.2113**	**1.874**	**0.090**

HCO_3^-* HCO_3^-	**-0.0000**	**-2.219**	**0.059**
pH*pH	**-0.3547**	**-9.644**	**0.00**
Co^{2+}* Co^{2+}	**-0.0064**	**-2.674**	**0.023**
HCO_3^-*pH	**-0.0003**	-2.246	**0.049**
HCO_3^-* Co^{2+}	0.0000	0.384	0.709
pH* Co^{2+}	-0.0073	-0.587	0.570

Linear model terms HCO_3^-, pH and Co^{2+}, Quadratic model terms $(HCO_3^-)^2$, $(pH)^2$ and $(Co^{2+})^2$ and interactive model terms (HCO_3^-, pH) were significant (p<0.05). While interactive model terms (HCO_3^-, Co^{2+}) and (pH, Co^{2+}) were not significant for the given response. Table 4 represents the ANOVA test for quadratic polynomial model. *P*-value of the model was significant at p<0.05. Higher values of R^2 (0.9656) have signified the validity of present model.

Table 4 Analysis of Variance (ANOVA) for quadratic polynomial model

Source	**Sum of Squares**	**Degree of freedom**	**Means Square**	**F value**
Regression	0.342155	9	0.038017	31.13[a]
Residual Error	0.012214	10	0.001221	-
Lack-of-Fit	0.011899	5	0.002380	-
Pure Error	0.000315	5	0.000063	-
Total	0.354369	19		-

[a] Statistical significance (α = 0.05)

Model is used to visualize the relationship between levels used for each factor and response in terms of dry weight, and also used to determine the interrelation between variables (Fig 4a-c). In Fig. 4a value of HCO_3^- was kept constant at maxima (1127 mg/l) and combined effect of pH and Co^{2+} was stimulated. Maximum dry weight was achieved when pH was at zero level and at highest concentration of Co^{2+}. When pH was held at maximum (8.76) highest dry weight was produced towards the higher concentrations of HCO_3^- and Co^{2+} (Fig. 4b). Whereas, at the maximum concentration of Co^{2+} (13.27 mg/l) the dry weight of biomass was highest at maximum concentration of HCO_3^- and zero level of pH (Fig. 4c).

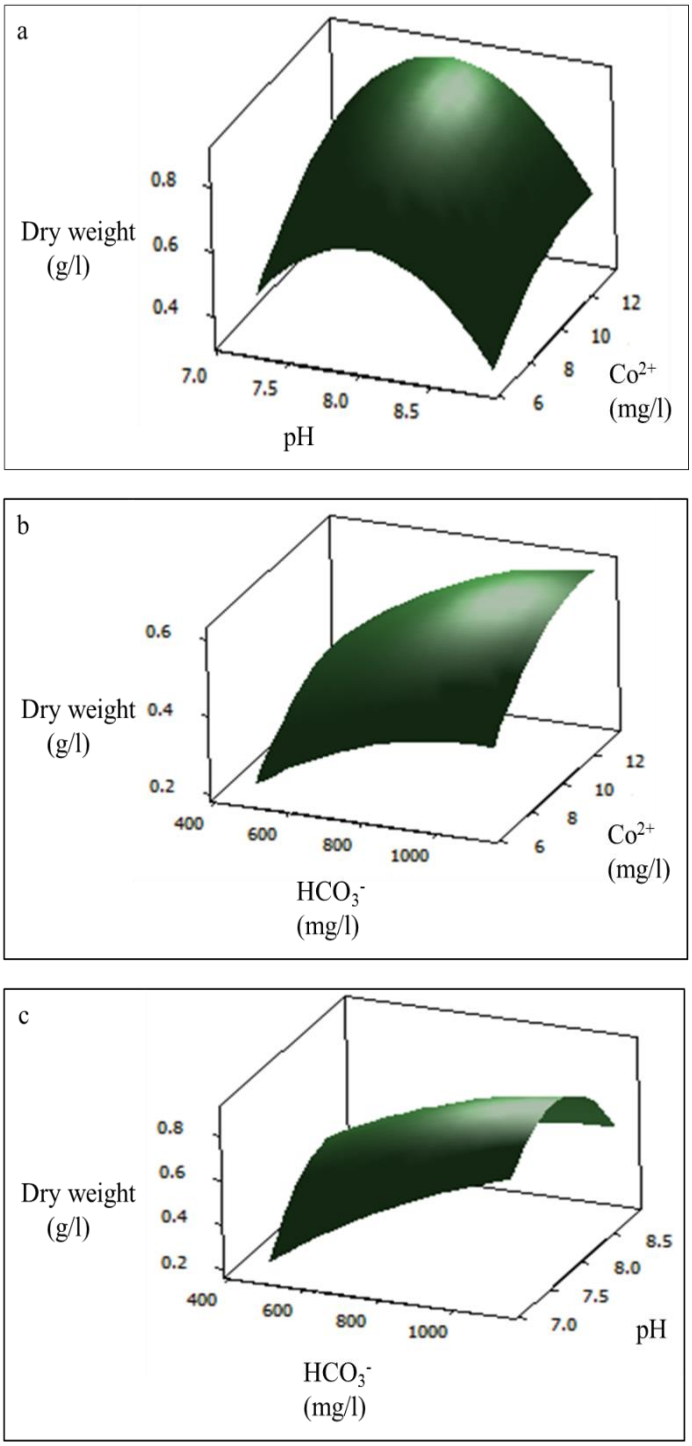

Fig. 4 Response surface plots representing interactive relationship between variables for response dry weight (mg/l) (a) Co^{2+} (mg/l) and pH (b) Co^{2+} (mg/l) and HCO_3^- (mg/l) (c) pH and HCO_3^- (mg/l)

When a response optimizer option was selected in Minitab® 16.2.2 software to obtain the maximum dry weight at an optimum condition from the predicted model, it gave the values as 1127 mg/l for HCO_3^-, 7.86 for pH and 13.07 mg/l for Co^{2+} and the highest predicted dry biomass was 0.891 g/l.

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