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Optimization of Process Parameters for CO_2 Fixation from Bicarbonate Source by a Microalgae

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ABSTRACT

Bicarbonate source for cultivation of microalgae is an alternate method mainly to avoid CO_2 loss. The main aim of the work is to assess the bio-fixation ability of CO_2 from ammonium bicarbonate by *Chlorella pyrenoidosa* under mixotrophic condition. Furthermore, statistical optimization has been carried out to study the influence of pH, concentration and inoculum size and identify best conditions for CO_2 removal. The results revealed that maximum removal of CO_2 was obtained at pH 6.0; with ammonium bicarbonate concentration, 6.66 g L^{-1} and inoculum size, 36.81%. The obtained results were statistically analyzed and the results were obtained with regression co-efficient R^2 value of 0.94 for CO_2 removal and 0.86 for corresponding chlorophyll content. From the study, it can be concluded that microalgae could able to grow in ammonium bicarbonate source which indicates that microalgae could assimilate ammonium and CO_2 in to their cells even at high concentration. Bicarbonate captured CO_2 proves to be a significant method for cultivation of microalgae supports commercial production.

Key words: Bicarbonate source, *Chlorella pyrenoidosa*, CO_2 removal, mixotrophic condition, statistical optimization

INTRODUCTION

Carbon dioxide is one of the important greenhouse gases responsible for global warming that are borne due to man-made activities especially from fossil fuel power plants (Zhuang *et al*., 2011; Chi *et al*., 2011; Borkenstein *et al*., 2011; Holloway, 2007). In order to overcome and effectively manage CO_2 emissions, various strategies have been adopted in power plants such as chemical absorption by using monoethanolamine (Pires *et al*., 2011), adsorption by activated carbon, sodium carbonate, zeolite (Wang *et al*., 2011), CO_2 as compressed gas (Metz, 2005), geological storage (Riemer, 1996), ocean storage (Stewart and Hessami, 2005), cryogenic liquefaction (Chiu *et al*., 2011), adsorption by molecular sieve and desiccant absorption (Jeong *et al*., 2003). However, some of the above processes have considerable effects on the environment (such as geological storage, ocean storage) and also the production, transportation and storage process (such as compressed gas, cryogenic liquefaction and chemical absorption) are not cost effective. On the other hand, biological capture (plants, microalgae) of CO_2 especially, microalgae has received a major attention as the microalgal biomass can also be used for production of commercial value added products and higher CO_2 fixation rates (Zhao and Su, 2014; Pires *et al*., 2012). Several studies have been conducted on CO_2 fixation of microalgae as alternative to the Carbon Capture Storage (CCS) technology

(Klinthong *et al*., 2015; Jacob-Lopes *et al*., 2008; Lam *et al*., 2012; Tang *et al*., 2011; Hsueh *et al*., 2009). However, the mass transfer of $CO_{2\,(g)}$ (i.e., gaseous form) in aqueous solution is very low, also the pH falls rapidly (Nayak *et al*., 2013; Kemmer, 1988). In order to overcome this issue, the carbonates produced from $CO_{2\,(g)}$ can be used as an alternative inorganic carbon source (Tang *et al*., 2011). Several studies have been conducted using sodium bicarbonate as an inorganic carbon source for the growth of microalgae (Moheimani, 2013; White *et al*., 2013; Aishvarya *et al*., 2012). Accordingly, this study has attempted to add to the current knowledge for carbon sequestration by microalgae from bicarbonate source. Based on the literature review, it has been identified that only few studies have been conducted with ammonium bicarbonate as an inorganic carbon source. Meanwhile, mixotrophic cultivation of microalgae has achieved higher growth rates and biomass (Abreu *et al*., 2012; Bhatnagar *et al*., 2011; Cheirsilp and Torpee, 2012). It is also envisaged that *Chlorella* sp. is capable of bio fixing HCO_3^- at mixotrophic conditions. Hence, in this study we aim to discuss the CO_2 removal by microalgae and to investigate the effects of process parameters such as pH, concentration of ammonium bicarbonate and inoculum size under mixotrophic mode. Furthermore, we have monitored chlorophyll as indicator for growth and photosynthetic productivity. The optimization experiments were designed based on central composite design using response surface methodology.

MATERIALS AND METHODS

Culturing of Microalgae: *Chlorella pyrenoidosa* (NCIM 2738) were purchased from the National Centre of Industrial Microorganism (NCIM), Pune, India and was cultured in Modified Bolds Basal Medium (UTEX., 2009) under sterile conditions. The stock solution was prepared individually for all the constituents in the media and the composition are as follows (g/100 mL): K_2HPO_4 0.4, $CaCl_2.2H_2O$ 0.36, $MgSO_4.7H_2O$ 0.75, $NaNO_3$ 15, citric acid 0.06, $Na_2EDTA.2H_2O$ 0.01, sodium carbonate 0.2, ammonium ferric citrate 0.06 and A_5 trace solution (g L^{-1}) components as H_3BO_3 2.86, $MnCl_2$ 1.81, $ZnSO_4.7H_2O$ 0.222, $Na_2MoO_4.2H_2O$ 0.390, $CuSO_4.5H_2O$ 0.079 and Co $(NO_3)_2.6H_2O$ 0.0494.

From the stock, the media have been prepared for 500 mL with 5 mL from each constituent with 0.5 mL of A_5 trace solution. The culture was subjected to continuous illumination with 1500 Lux measured using a TES light meter (TES CORP).

Experimental design: The exponential phase microalgae cells were taken for experimental studies in synthetic medium (Feng *et al*., 2011) with the following composition (g L^{-1}); glucose 0.4125, NH_4Cl 0.078, KH_2PO_4 0.018, $MgSO_4.7H_2O$ 0.013, $CaCl_2.2H_2O$ 0.043, $FeSO_4.7H_2O$ 0.005; A_5 trace solution (1 mL L^{-1}), respectively. All the experiments in the study was carried in 500 mL conical flasks containing 300 mL of working solution of synthetic medium with variables (pH, inoculum size and ammonium bicarbonate concentration). The pH values were chosen to study the microalgae behavior to CO_2 in acidic (pH 4 to mimic more availability of free CO_2), pH 6 (as standard growth medium range) and alkaline range (pH 8). The ammonium bicarbonate concentrations were fixed of 1-3 g/300 mL each which was scaled up in g L^{-1} as depicted in Table 1. The inoculum sizes were fixed (10-30%) on volume per volume basis as to envisage its effects for CO_2 removal. All the flasks were manually shaken thrice a day in order to avoid sticking of culture to flasks.

Response surface methodology: In order to study the effects of the chosen variables (pH, inoculum size and NH_5CO_3 concentration) on the maximum removal of CO_2 and chlorophyll

Table 1: Experimental range and levels of independent variables

Independent variables	Design variables	Range and levels		
		-1	0	1
pH	A	4	6	8
Ammonium bicarbonate (g L^{-1})	B	3.33	6.66	10
Inoculum size (%)	C	10	20	30

Table 2: Full factorial central composite design matrix with code and real variables

Run	A	B	C	pH	NH_5CO_3 (g L^{-1})	Inoculum size (%)
1	-1	-1	-1	4.0	3.33	10
2	1	-1	-1	8.0	3.33	10
3	-1	1	-1	4.0	10	10
4	1	1	-1	8.0	10	10
5	-1	-1	1	4.0	3.33	30
6	1	-1	1	8.0	3.33	30
7	-1	1	1	4.0	10	30
8	1	1	1	8.0	10	30
9	-1.68179	0	0	2.6	6.66	20
10	1.68179	0	0	9.4	6.66	20
11	0	-1.68179	0	6.0	1.06	20
12	0	1.68179	0	6.0	12.27	20
13	0	0	-1.68179	6.0	6.66	3.18
14	0	0	1.68179	6.0	6.66	36.81
15	0	0	0	6.0	6.66	20
16	0	0	0	6.0	6.66	20
17	0	0	0	6.0	6.66	20
18	0	0	0	6.0	6.66	20
19	0	0	0	6.0	6.66	20
20	0	0	0	6.0	6.66	20

content, 20 sets of experiments with appropriate combinations of pH, inoculum size and ammonium bicarbonate concentration based on 3 factors of 2^3 central composite factorial design were conducted using response surface methods (statistical analysis) and the details are presented in Table 2. The Central Composite Design (CCD) is employed in order to show the nature of the response surface in the experimental design and to elucidate the optimal conditions of the most significant independent variables. In this analysis, ammonium bicarbonate concentration, inoculum size and pH were chosen as independent variables and the carbon dioxide (CO_2) removal rate (%) and its corresponding chlorophyll content was taken as dependent output response variable. All the experiments in the study were carried in 500 mL conical flasks containing 300 mL of working solution of synthetic wastewater medium with ammonium bicarbonate (NH_5CO_3) concentration in the range of 1-3 g/300 mL each, which was scaled up in g L^{-1} as depicted in Table 1. The culture was subjected to continuous illumination with 1500 Lux measured using a TES light meter (TES CORP). All the flasks were manually stirred thrice a day in order to avoid sticking of culture to flasks. The chlorophyll and CO_2 estimation (by forms of alkalinity) has been carried for every day i.e., at 24 h interval until the stationery phase has been achieved.

The three independent variables were varied over 2 levels with pH between (4 and 8) relative to the center point (pH 6), the second independent variable (ammonium bicarbonate in g L^{-1}) was varied over two levels (3.3 and 9.9 g L^{-1}) relative to the center point (6.6 g L^{-1}) and the third independent variable (inoculum size in %) was varied over two levels (10 and 30%) relative to the center point (20%).

The full factorial central composite design matrices of three variables with respect to their uncoded (real) and coded values are presented in Table 2. The response surface method was constructed using MINITAB 16 statistical software. Evaluation of the goodness of fit of the model is done through coefficient determination and analysis of variances. The experimental results were fitted to a second order polynomial Eq. 1:

$$Y = \beta_0+\beta_1A+\beta_2B+\beta_3C+\beta_{11}A^2+\beta_{22}B^2+\beta_{33}C^2+\beta_{12}AB+\beta_{13}AC+\beta_{23}BC \quad (1)$$

where, Y is the dependent variable (CO_2 removal and chlorophyll content), A, B and C are the independent variable, β_0 is the regression coefficient at center point, β_1, β_2 and β_3 are the linear coefficients, β_{11}, β_{22} and β_{33} are the quadratic coefficients and β_{12}, β_{13} and β_{23} are the second order interaction coefficients. The developed regression model was evaluated by analyzing the values of regression coefficients, analysis of variance (ANOVA), p and F-values. The quality of fit of the polynomial model equation was expressed with the coefficient of determination, R^2. The statistical software package was used to identify the experimental design as well as to generate a regression model to predict the optimum combinations considering the effects of linear, quadratic and interactive effects on CO_2 removal and corresponding chlorophyll content.

Carbon dioxide removal rate: When ammonium bicarbonate is dissolved in water, it will be in various forms of alkalinity as represented in the following equation (Van den Hende *et al*., 2012; Zhuang *et al*., 2012):

$$NH_4HCO_3 \rightarrow NH_3+CO_2+H_2O \quad (2)$$

$$NH_3+H_2O \rightarrow NH_4^+ +OH^- \quad (3)$$

$$CO_{2\,(a\text{-}q)}+H_2O \rightarrow H_2CO_3^* \rightarrow HCO_3+H^+ \rightarrow CO_3^{2-} + 2H^+ \quad (4)$$

$$H_2CO^*_3 \rightarrow CO_{2\,(aq)}+H_2CO_3$$

Where:
$H_2CO^*_3$ = Free CO_2 (as mg $CaCO_3$/L)
HCO_3 = Bicarbonate alkalinity(as mg $CaCO_3$/L)
CO_3 = Carbonate alkalinity(as mg $CaCO_3$/L)

The removal of CO_2 was calculated by different forms of alkalinity species (HCO_3^-, CO_3^{2-}, $H_2CO_3^*$) which is measured by following the standard methods prescribed by American Public Health Association (APHA., 2005). The sum of these alkalinity species is total inorganic carbon and is expressed as CO_2 (as CaCO3) (Eq. 5). The empirical relation (Kemmer, 1988) was used to calculate CO_2 (as mg L^{-1}) from TIC and is given in Eq. 6:

$$CO_{2\,(as\,CaCO_3)}\,(mg\,L^{-1}) = H_2CO_3^* + HCO_3 + CO_3^{2-} \quad (5)$$

$$CO_{2\,(as\,CaCO_3)}\,(mg\,L^{-1}) \div 1.14 = CO_{2\,(as\,CO_2)}\,(mg\,L^{-1}) \quad (6)$$

The CO_2 removal (%) was determined by calculating difference between the initial concentration of CO_2 (based on Eq. 6) from each experimental runs and final concentration after growth in stationery phase (until no observation of CO_2 removal was found) and is expressed in Eq. 7:

$$CO_2\ \text{removal}(\%) = \frac{\text{Initial}\,CO_2 - \text{Final}\,CO_2}{\text{Initial}\,CO_2} \times 100 \qquad (7)$$

Chlorophyll analysis: The chlorophyll content in the medium is determined by spectrometric analysis (Becker, 1994). Five milliliter algae culture was centrifuged at 10000 rpm for 10 min. The supernatant was drained off and the sample was re-suspended in ethanol/diethyl ether and kept boiling for 5 min. After boiling, the sample was made up to 5 mL with ethanol/diethyl ether. The optical density was measured at 660 and 642.5 nm with solvent as a blank. The chlorophyll content was determined using the equation:

$$\text{Chlorophyll (mg L}^{-1}) = (9.9\times OD_{660})\text{-}(0.77\times OD_{642.5})$$

RESULTS AND DISCUSSION

Statistical analysis

CCD: The results of CO_2 removal and chlorophyll content (both predicted and experimental) for different design variables (pH, inoculum size and Ammonium bicarbonate concentration) are presented in the Table 3. The influence of three chosen variables (pH, ammonium bicarbonate concentration and inoculum size) was fitted in to the second order polynomial Eq. 1. The fitted second order polynomial equation for maximum CO_2 removal and chlorophyll content (mg L^{-1}) are shown in Eq. 8 and 9:

CO_2 removal (%):

$$Y = 60.187+6.2324A-0.9728B+3.8726C-5.7662A^2-3.1340B^2+0.3980C^2-4.2237AB-2.8063AC-1.2063BC \qquad (8)$$

Table 3: Central composite design matrix and the output responses for CO_2 removal and chlorophyll

Run	pH	NH_5CO_3 (g L^{-1})	Inoculum size (%)	CO_2 removal (%) (experimental)	CO_2 removal (%) (predicted)	Chlorophyll (mg L^{-1}) (experimental)	Chlorophyll (mg L^{-1}) (predicted)
1	4.0	3.33	10	33.33	34.3166	1.96	1.43328
2	8.0	3.33	10	63.07	60.8413	0.95	1.71610
3	4.0	10.00	10	40.00	43.2309	4.76	4.47138
4	8.0	10.00	10	51.00	52.8607	0.08	0.66920
5	4.0	3.33	30	50.00	50.0868	1.81	1.25000
6	8.0	3.33	30	66.67	65.3866	1.51	1.82783
7	4.0	10.00	30	50.00	54.1762	5.29	4.55310
8	8.0	10.00	30	51.62	52.5809	0.49	1.04592
9	2.6	6.66	20	37.50	33.3963	0.30	1.57002
10	9.4	6.66	20	53.01	54.3595	0.17	-1.14133
11	6.0	1.06	20	50.57	52.9590	2.83	2.84573
12	6.0	12.27	20	54.83	49.6868	4.80	4.74296
13	6.0	6.66	3.18	56.15	54.8000	2.41	2.10301
14	6.0	6.66	36.81	69.23	67.8259	2.00	2.26568
15	6.0	6.66	20	60.00	60.1871	1.15	1.13285
16	6.0	6.66	20	60.12	60.1871	1.13	1.13285
17	6.0	6.66	20	60.14	60.1871	1.12	1.13285
18	6.0	6.66	20	60.12	60.1871	1.14	1.13285
19	6.0	6.66	20	60.13	60.1871	1.13	1.13285
20	6.0	6.66	20	60.14	60.1871	1.12	1.13285

Chlorophyll (mg L^{-1}):

$$Y = 1.13285-0.80609A+0.56405B+0.04836C-0.32475A^2+0.94098B^2+0.37176C^2-1.02125AB+0.07375AC-0.06625BC \quad (9)$$

ANOVA has been used to analyze the significance of the second order polynomial equation for the dependent variables ANOVA values obtained from the CCD model for the quadratic regression model has been shown in the Table 4 (CO_2 removal) and Table 5 (chlorophyll). F-Test was carried out to compare the model for degree of adequacy. If the model has a high degree of adequacy for predicting the experimental results, the computed F-value from the model should be greater than the F tabulated value (Demirel and Kayan, 2012). From the Table 4 and 5, the obtained F-value for CO_2 removal and chlorophyll are 18.35 and 7.05 which is greater than F tabulated value (3.02). The regression co-efficient (R^2) results obtained for maximum removal of CO_2 and chlorophyll content are 0.94 and 0.86 which shows the high degree of correlation between experimental and predicted values. The estimated regression coefficients for removal of CO_2 and chlorophyll content are presented in Table 6, respectively, along with their corresponding p-value and t-values. The p-value is used to check the interactive effects of each independent variable. It can be observed from Table 6 for CO_2 removal (%) that, the coefficient for single effect of pH (β1) and inoculum size (β3) are highly significant ($p<0.050$) whereas, the square effects i.e., linear co-efficient β33 and the interactive terms β23 were not significant while for chlorophyll (mg L^{-1}) the coefficient except the inoculum size (β3) for single effect is not significant whereas for interactive and square effects such as, were not significant.

Table 4: ANOVA for fit of CO_2 removal (%) from central composite design

Sources of variation	Sum of squares	Degree of freedom	Mean square	F-value	p-value
Regression	1561.47	9	173.490	18.35	0.0000
Residuals	94.53	10	9.453		
Total	1655.94				
R^2 = 94.29%	$R_{2\,(adjusted)}$ = 89.15%				

Table 5: ANOVA for fit of chlorophyll (mg L^{-1}) from central composite design

Sources of variation	Sum of squares	Degree of freedom	Mean square	F-value	p-value
Regression	38.4449	9	4.2717	7.05	0.003
Residuals	6.0624	10	0.6063		
Total	44.508				
R^2 = 86.38%	$R_{2\,(adjusted)}$ = 74.12%				

Table 6: Estimated regression coefficients for CO_2 removal (%) and chlorophyll (mg L^{-1})

	Coefficient		Standard error		T		p	
Term	CO_2 removal (%)	Chlorophyll a (mg L^{-1})	CO_2 removal (%)	Chlorophyll a (mg L^{-1})	CO_2 removal (%)	Chlorophyll a (mg L^{-1})	CO_2 removal (%)	Chlorophyll a (mg L^{-1})
β_0	60.1871	1.13285	1.2539	0.3176	47.999	3.567	0.000	0.005
β_1	6.2324	-0.80609	0.8320	0.2107	7.491	-3.826	0.000	0.003
β_2	-0.9728	0.56405	0.8320	0.2107	-1.169	2.677	0.269	0.023
β_3	3.8726	0.04836	0.8320	0.2107	4.655	0.230	0.001	0.823
β_{11}	-5.7662	-0.32474	0.8099	0.2051	-7.120	-1.583	0.000	0.144
β_{22}	-3.1340	0.94098	0.8099	0.2051	-3.870	4.588	0.003	0.001
β_{33}	0.3980	0.37176	0.8099	0.2051	0.491	1.812	0.634	0.100
β_{12}	-4.2237	-1.02125	1.0870	0.2753	-3.886	-3.710	0.003	0.004
β_{13}	-2.8063	0.07375	1.0870	0.2753	-2.582	0.268	0.027	0.794
β_{23}	-1.2063	0.06625	1.0870	0.2753	-1.110	0.241	0.293	0.815

Effect of variables on CO_2 removal: The main objective of the response surface method is to find out the optimum condition for maximum CO_2 removal with respect to the chosen variables. The interaction effects between the variables (pH, ammonium bicarbonate and inoculum size) for carbon dioxide removal are presented in the Fig. 1. Figure 1a shows the interaction effects between the pH and ammonium bicarbonate. It can be noticed that as the pH increases to high value with low ammonium bicarbonate concentration the removal of CO_2 was high, whereas at higher pH and increased concentration of ammonium bicarbonate, the removal of CO_2 decreases. These are due to availability of HCO_3 ions for microalgae that uptake and fix CO_2 by carbonic anhydrase activity (Zhao and Su, 2014). From the Fig. 1b, it is seen that, as the pH increases with low inoculum size there was a maximum removal of CO_2 whereas, as the pH attains high value with increase in inoculum size, no change was observed and it is clear that all the bicarbonate ions are well utilized by microalgae and the linear trend observed may be due to formation of hydroxides (Nayak *et al*., 2013) but when pH increases at high inoculum size we can observe that the CO_2 removal was high compared to level low inoculum size this shows that the more the inoculum size more the CO_2 removal. From the Fig. 1c the interaction effect between ammonium bicarbonate and inoculum size has been depicted. It has been observed that in the range of middle values and low inoculum size

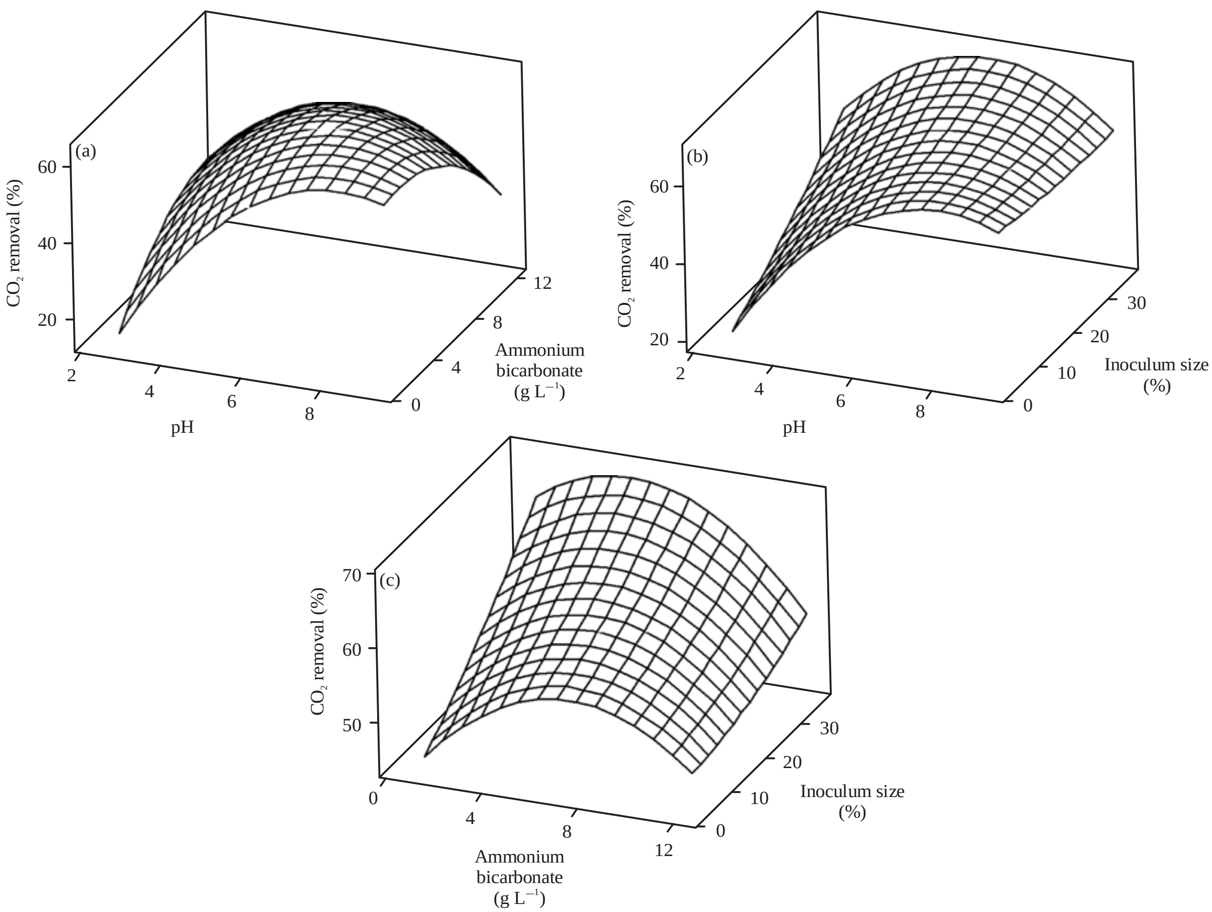

Fig. 1(a-c): Surface plot for CO_2 removal, (a) Concentration of NH_5CO_3, pH, (b) Inoculum size, pH and (c) Inoculum size, Concentration of NH_5CO_3

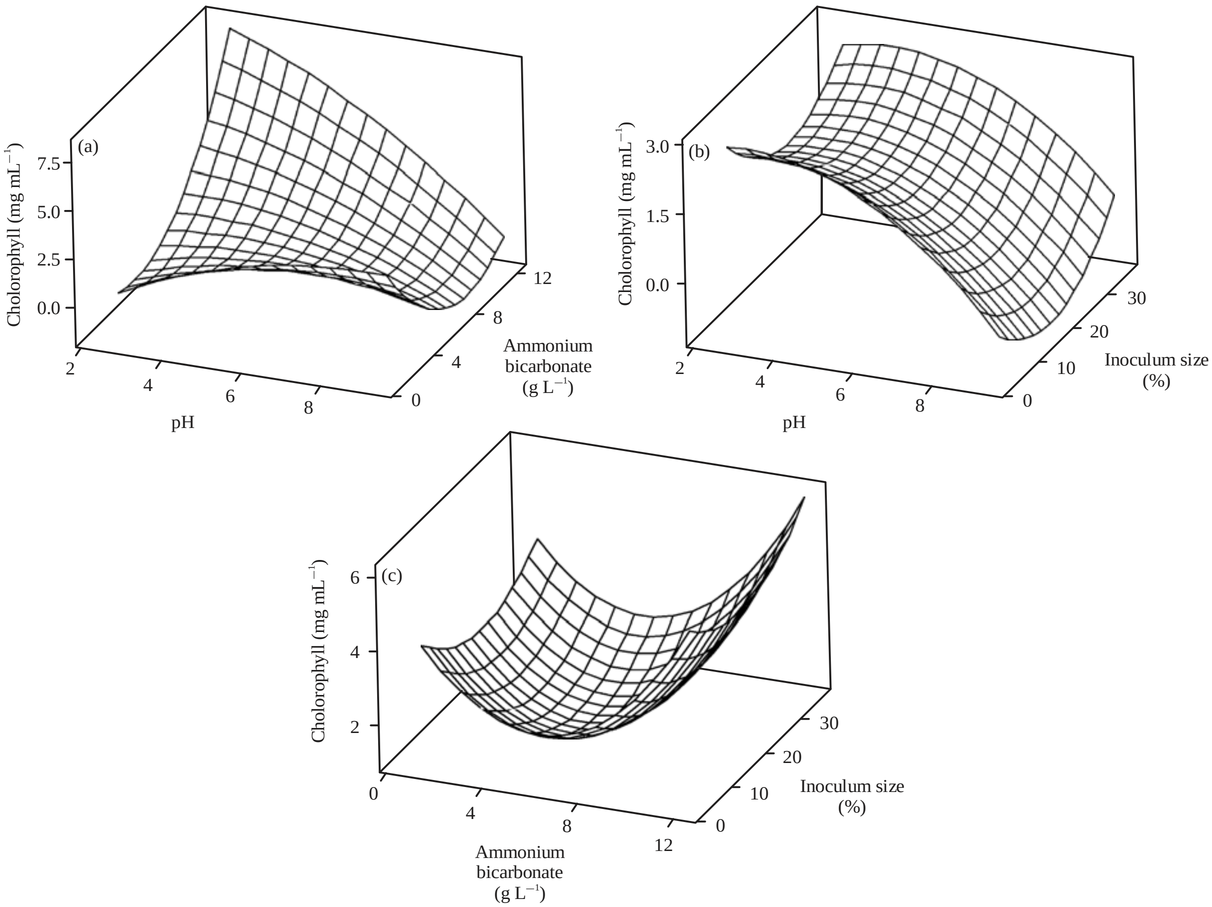

Fig. 2(a-c): Surface plot for chlorophyll, (a) Concentration of NH_5CO_3, pH, (b) Inoculum size, pH and (c) Inoculum size, Concentration of NH_5CO_3

the CO_2 removal increases whereas, at high inoculum size the removal of CO_2 was very high compared to low inoculum size this shows that increase in inoculum size leads to more uptake of HCO_3 ions from the medium(Van den Hende *et al*., 2012).

Effect of variables on chlorophyll content: Chlorophyll "a", pigment present in microalgae is produced during photosynthesis process as in Eq. 10:

$$6CO_2+6H_2O + h\upsilon \text{ (sunlight)} \rightarrow C_6H_{12}O_6 \quad (10)$$

Chlorophyll is an essential component of photosynthesis in green microalgae that captures light energy and CO_2 for metabolic activity for cell growth and lipid accumulation (Li *et al*., 2008). The interaction effect between the variables (pH, ammonium bicarbonate and inoculum size) on chlorophyll content of microalgae has been shown in the Fig. 2. The effect of pH and ammonium carbonate on chlorophyll content has been shown in the Fig. 2a. It can be noticed from the Fig. 2a that as the pH increases at an initial ammonium bicarbonate concentration the chlorophyll content increases but the reaction was vice versa when the concentration of ammonium bicarbonate increases i.e., there was a decrease in chlorophyll content when the ammonium bicarbonate concentration increases at high level of pH (Ho *et al*., 2011; Van den Hende *et al*., 2012). This could be due to the fact that when the pH increases, the ammonium level decreases. Also from the figure, it can be observed that at higher ammonium bicarbonate concentration and low pH, the chlorophyll content is high which is due to the presence of more ammonium ions at lower pH. Figure 2b depicts the effect of pH and inoculum size, in which, it can be observed that at a low inoculum size when the pH increases the chlorophyll content decreases and the same pattern has been observed at a high inoculum size which is due to maximum uptake of HCO_3 by the cell such that regulation of H^+ ions that leads to formation of hydroxides thus decreases the chlorophyll content (Nayak *et al*., 2013). The effect of ammonium bicarbonate and inoculum size has been depicted in Fig. 2c. It can be noticed that, as the ammonium bicarbonate concentration increases there is a decreasing trend initially and the chlorophyll content increases later. This could be due to the fact that the initially the cells were in exponential phase so that at initial level there was more chlorophyll content. The decrease in chlorophyll content could be due to the adaptation of microalgae in the medium. At later stage, chlorophyll starts increasing which may be due to the fact that when large source of nitrogen compound is available, the cells will produce large chlorophyll content (Li *et al*., 2008).

CONCLUSION

This study indicates the feasibility of the use of NH_5CO_3 as a potential inorganic carbon source for growth of microalgae under various process conditions. The effects of pH, inoculum size and ammonium bicarbonate concentration on CO_2 removal using *Chlorella pyrenoidosa* in wastewater medium was investigated by employing the response surface method. The maximum removal of CO_2 (69%) was achieved at pH 6 with ammonium bicarbonate concentration of 6.66g L^{-1} and inoculum size of 36.8% and the corresponding chlorophyll content is 2 mg L^{-1}. The regression coefficient (R^2) of 0.94 and 0.86 was observed for CO_2 removal and chlorophyll content which implies that the experimental results are statistically significant. Based on the investigation, it has been identified that *Chlorella pyrenoidosa* has great potential for CO_2biofixation.

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