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Full Length Research Paper

Optimization of carbon dioxide fixation and starch accumulation by *Tetraselmis subcordiformis* in a rectangular airlift photobioreactor

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Culture conditions are very important to CO_2 bio-fixation and carbohydrate accumulation in microalgae. The objective of this study was to optimize semi-continuous culture conditions of *Tetraselmis subcordiformis* in a rectangular airlift photobioreactor for obtaining maximized carbon dioxide fixation rate and intracellular starch productivity. The effects of the initial biomass concentration (0.13, 0.8, 1.8, 2.8 and 3.47 g L^{-1}), the gas flow rate (0.03, 0.1, 0.2, 0.3 and 0.37 V V^{-1} m^{-1}) and different carbon dioxide concentrations (1.63, 5, 10, 15 and 18.37%) were considered using a central composite design. By using response surface methodology and the desirability function approach, the optimal CO_2 fixation rate of 55.15 mg L^{-1} h^{-1} and the intracellular starch productivity of 10.66 mg L^{-1} h^{-1} occurred when the initial biomass concentration was 1120 mg L^{-1} and the air supplemental CO_2 concentration was 6.9% with a gas flow rate of 0.35 V V^{-1} m^{-1}. These results suggest the potential of applying *T. subcordiform* to CO_2 mitigation and starch production.

Key words: Photobioreactor, CO_2 fixation rate, starch productivity, response surface methodology, desirability function approach.

INTRODUCTION

Global warming induced by increasing concentrations of greenhouse gases in the atmosphere is causing more and more concern. CO_2 is the main greenhouse gas, and 28.8 billion tons of it was released into the atmosphere in 2007 alone (International Energy Agency, 2009). The IEA predicted this number would reach to 34.5 billion tons or more by the year 2020. During the last two decades, various CO_2 mitigation strategies were investigated that have included chemical reaction-based approaches, renewable biological energy sources, CO_2 fixation and terrestrial sequestration of carbon, among others. At present, a large amount of research suggests that, using microalgae for CO2 fixation is a sustainable strategy because microalgae have much higher growth rates and CO_2 fixation abilities than conventional terrestrial plants. They can incorporate CO_2 into cells in the form of biopolymers such as carbohydrates, proteins, lipids and other valuable products by photosynthetic reactions (Chiu et al., 2008; Hsueh et al., 2009). In addition, a recent report (Ramanan et al., 2010) indicated that *Chlorella sp.* and *Spirulina platensis* were able to deposit CO_2 in the form of calcite with CO_2 fixation photosynthetically occurring at the same time. This seems a viable option in the efforts to sequester increasing CO_2 emissions.

Starch is one of the main biopolymers that accumulate intracellularly in unicellular green algae and it can be converted to hydrogen and alcohol. Several groups that have focused on microalgal hydrogen production found that intracellular starch content markedly effected hydrogen

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production and they proposed approaches to increase productivity of hydrogen by engineering starch metabolism (Burrows et al., 2008; Guo et al., 2008; Mathews and Wang, 2009). In addition, using microalgal starch as a fermentation feedstock for bio-ethanol production displays advantages over other traditional feedstocks (Hirayama et al., 1998; Harun et al., 2010). Matsumoto et al. (2003) used marine bacterial amylase for saccharification of the microalgae NKG 120701, which contain considerable amounts of intracellular carbohydrate. This research suggested that high starch content in algal biomass led to a high potential for alcohol production.

Cultivation conditions such as light regime, gas flow rate in bioreactors and environmental CO_2 concentration are very important factors in microalgae growth and intracellular substance accumulation (Bartual and Gálvez, 2002; Yoon et al., 2008). Hence, choosing an appropriate experimental design for optimization of cultivation conditions will help in obtaining more algal biomass and achieving higher CO_2 fixation levels (Jacob-Lopes et al., 2008). In numerous experimental design methods, response surface methodology (RSM) has been comprehensively applied in many scientific research fields for its minimal amount of experiments required and effective process optimization. However, when a system has several response variables needing simultaneous optimization, RSM often becomes unsatisfactory. This is because what is optimal for one response variable may not be optimal for another. The desirability function approach (DFA), a method widely used in separation and analysis, but rarely in microalgae cultivation research, can be introduced to make up for this deficiency by transforming two or more response variables to overall desirability (*D*) (Oddone et al., 2007; Li et al., 2007). Thus, it is a good choice to make DFA an extension of RSM in order to find the best compromises in optimizing algal cultivation conditions.

In the course of optimizing operational conditions, the characteristic parameters (response variables) investigated within a culture system mainly focus on biomass productivity, lipid accumulation rate and protein synthesis rate, but a few focus on the evaluation of intracellular starch productivity in the system (Chiu et al., 2009; Mandal and Mallick, 2009). For the purpose of investigating CO_2 fixation rates and algal starch productivity, a rectangular airlift photobioreactor that had inside baffles to improve the medium mixing was developed, then a five level central composite design was used to evaluate the relationship between the independent variables (initial biomass density, gas flow rate and CO_2 concentration) and dependent variables (CO_2 fixation rate and starch productivity).

The aim of this work was the optimization of culture conditions under which optimal CO_2 fixation rates and intracellular starch productivity occur simultaneously by using RSM and the DFA. *Tetraselmis subcordiformis*, a marine green microalga that contains considerable amounts of starch, was applied to all sets of experiments.

MATERIALS AND METHODS

Microorganism and medium

T. subcordiform, a marine green microalga, was kindly presented by the Institute of Aquaculture of Liaoning Province, Dalian, China. The microalgae were cultivated in modified Walne medium in natural seawater that had the following composition (per liter): 1.3 mg $FeCl_3$, 0.36 mg $MnCl_2$, 33.6 mg H_3BO_3, 45 mg EDTA, 50 mg NaH_2PO_4, 100 mg $NaNO_3$, 0.21 mg $ZnCl_2$, 0.2 mg $CoCl_2$, 0.09 mg $(NH_4)_4Mo_7O_{24}$, 0.2 mg $CuSO_4$, 0.1 µg vitamin B12 and 1 µg vitamin B1, pH 8.0.

Photobioreactor system for culture

In this study, a rectangular airlift photobioreactor that had inside baffles to improve the medium mixing was developed. The system was constructed with plexiglass plate, with height of 1200 mm, length of 115 mm, width of 90 mm and a working volume of 10.5 L. There were two slots to place the 960 mm high baffle plates. The slots were located 21.5 mm away from the two reactor walls, respectively. Illumination from the two sides of the bioreactor was provided by 20 cool white fluorescence tubes with continuous light at a photon flux density (PFD) of 150 µE m^{-2} s^{-1}. The average light density was measured on six different sites on the bioreactor surface by a photosynthetically active radiation (PAR) detector (Optometer P9710 with PAR detector 3701, Gigahertz Optik Corporation, Germany). The cultivation temperature was controlled at 25 ± 1°C by a thermostatic water bath. Air flow into the photobioreactor was provided via filtered air and a pure CO_2 cylinder through a porous polyester sparger located at the bottom of the system. The flow rates of the CO_2/air mixture and desired CO_2 concentration were adjusted through three rotameters and calibrated using a gas chromatograph (SRI8610, SRI Instruments, USA) with TCD detector. The details of the system and schematic diagram are shown in Figure 1.

Experimental design and response surface analysis

In order to investigate the effects of the cultivation conditions (initial biomass concentration, gas flow rate and CO_2 concentration) on the CO_2 fixation rate and starch productivity, a five-level central composite design was introduced that contained 17 experiments and three replicates at the central point, that were used to estimate the experimental error. The independent variable levels X_i were coded as x_i according to Equation 1:

$$x_i = \frac{X_i - X_0}{\Delta X_i}, i = 1, 2, 3, ..., k, \quad (1)$$

Where X_i and x_i are the actual and the coded value, respectively, X_0 is the value of an independent variable at center point and ΔX_i is the step change. Table 1 shows the levels of the experimental variables used.

Through response surface analysis, a second-order model fit to the response of the independent variables was constructed. The fitting model can evaluate the results of single-parameter effects up to the second order and all binary interactions and calculate optimal independent variable conditions for obtaining the desired response.

Equation 2 gives the general form of such a model:

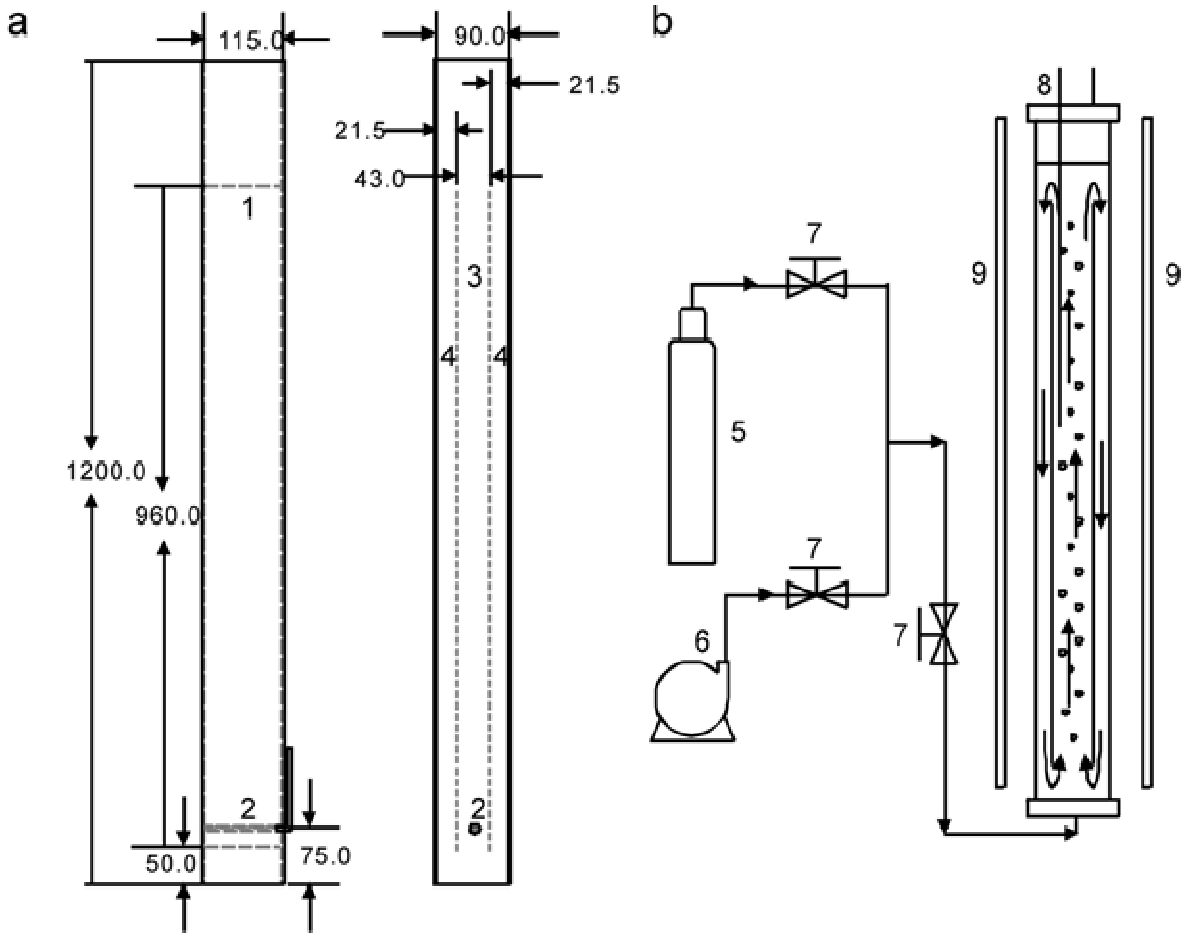

Figure 1. Photobioreactor diagram. (a), Design dimension of the bioreactor; (b), bioreactor procedure scheme. 1, inside baffle; 2, gas sparger; 3, riser; 4, downcomer; 5, CO_2 gas cylinder; 6, air compressor; 7, rotameter; 8, bioreactor; 9 , fluorescent lamp.

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \sum_{j=1}^{k} \beta_{i,j} x_i x_j \quad (2)$$

Where, Y is the predicted response, β represents a regression coefficient, x_i and x_j are the input variables that influence the response variable and k is the number of factors. In this study, the k value was equal to three. The experimental design and the statistical analyses were carried out using Statistica 8.0 software (Statsoft, USA).

Simultaneous optimization of two response variables

When a system has several response variables needing simultaneous optimization, RSM often becomes unsatisfactory in solving this kind of problem. The desirability function approach (DFA) can be introduced to make up for this deficiency. In the DFA, the multicriteria problem is reduced to a single criterion problem of D optimization by transforming two or more response variables to the overall desirability (Derringer and Suich, 1980). D is defined as follows in Equation 3:

$$D = \left(\prod_{i=1}^{n} d_i \right)^{1/n} \quad (3)$$

Where, n is the number of responses to be included in the optimization and d_i is the individual desirability of response i. In this study, a one-sided transformation was used to transform individual responses into a corresponding desirability value. Equation 4 defined the individual desirability (d_i):

$$d_i = \begin{cases} 0 & Y_i \le Y_{i*} \\ \left[\frac{Y_i - Y_{i*}}{Y_i^* - Y_{i*}} \right]^r & Y_{i*} < Y_i < Y_i^* \\ 1 & Y_i \ge Y_i^* \end{cases} \quad (4)$$

Where, Y_i is response i, Y^*_i and Y_{i*} are the highest and lowest

Table 1. Values of the independent variables in the different levels of the design.

Independent variable	Symbol	Level				
		-1.68	-1	0	+1	+1.68
Initial biomass concentration (mg L^{-1})	X_1	130	800	1800	2800	3470
Gas flow rate (V V^{-1} m^{-1})	X_2	0.03	0.1	0.2	0.3	0.37
CO_2 concentration (V/V %)	X_3	1.63	5	10	15	18.37

experimental values of Y_i, and r is a user-specifiable weight. In this case, r was set to 1, making d_i a linear function to a certain response. The individual desirability (d_i) for any experimental response value therefore ranges between 0, for the lowest observed value and 1, for the highest. According to Equations 3 and 4, it can be deduced that, the overall desirability (D) is a continuous function of Y_i. As a result, maximization of D over the range of independent variables can be realized by using the existing univariate search techniques. Once the maximum value of D is found, the optimal operational conditions (independent variables) are determined, achieving the purpose of simultaneous optimization.

Semi-continuous cultivation and biomass measurement

The cultures were operated on a semi-continuous mode. An appropriated volume of culture was harvested once every 12 h and replaced by the same volume of fresh culture in order to maintain the experimental initial biomass concentration. The average increase in biomass during 72 h was used to evaluate the CO_2 fixation rate and starch productivity.

A previous study indicated a significant correlation (R^2 = 0.996) between the culture's OD_{625} and the biomass concentration (g L^{-1}), and that relationship was: biomass concentration = 0.5532×OD_{625}. Therefore, the values of OD_{625} were used to calculate the biomass concentration. The OD_{625} was measured by a Jasco V-530 UV/Visible spectrophotometer (Jasco Corporation, UK). The specific growth rate (μ, h^{-1}) was calculated according to Equation 5:

$$\mu = \frac{\ln X_2 - \ln X_1}{t_2 - t_1} \quad (5)$$

Where X_1 and X_2 are the biomass concentration (dry weight) at times t_1 and t_2, respectively (Lee and Shen, 2004).

Analytical methods

The elemental composition of the *T. subcordiform* cells was determined using a Vario EL III CHNS element analyzer (ELEMENTAR Corporation, Germany). 2 ml samples of biomass were oxidized at 1000°C and the resulting gases were determined using a thermal conductivity detector for carbon, nitrogen and hydrogen. The standard used was acetanilide with a composition of 71.09% carbon, 11.84% oxygen, 6.71% hydrogen and 10.36% nitrogen.

The CO_2 fixation rate (R_C) was calculated from an elemental analysis of the algal biomass, as shown in Equation 6:

$$R_C = C_c \times \left(\frac{X_2 - X_1}{t_2 - t_1} \right) \times \left(\frac{M_{CO_2}}{M_C} \right) \quad (6)$$

Where, C_c is the carbon content of the biomass and M_{CO2} and M_C are the molecular weight of CO_2 and carbon, respectively.

For determining starch content (S_c), 5 mg sample of algae powder was suspended in 5 ml 0.1 N sodium acetate. After disruption in an ultrasonic disintegrator (400 W, 20 cycles of 15 s; JY92-II, Ningbo Scientz Biotechnology Co. Ltd, Ningbo, China), the suspension was centrifuged (4,000 g, 10 min; ROTINA46 Hettich, Tuttlingen, Germany) and the pellet was used for the following steps. Starch was extracted by boiling (for 30 min) in 5 ml of distilled water (Klein and Betz, 1978). The starch levels were determined using the amyloglucosidase assay kit (Sigma-Aldrich, St. Louis, MO, USA). The starch productivity (P_S) was then be calculated according to Equation 7:

$$P_S = S_c \times \left(\frac{X_2 - X_1}{t_2 - t_1} \right) \quad (7)$$

RESULTS AND DISCUSSION

The effect of operational conditions on *T. subcordiform* growth and CO_2 utilization efficiency

To investigate the effect of different cultivation conditions on *T.subcordiform* growth and CO_2 utilization efficiency, samples were gathered once every 12 h and biomass concentration was determined by measuring the OD_{625} value. Table 2 summarizes the results of the growth parameters and response values. The biomass productivity ranged from 10.01 to 34.17 mg $L^{-1}h^{-1}$, demonstrating that the initial biomass concentration, gas flow rate and CO_2 concentration distinctly affected *T. subcordiform* growth. The light conditions in the bioreactor were strongly influenced by biomass concentration and "mutual shading" or "light inhibition" occurred when the biomass concentration was excessively high or low, respectively. There was some difference in biomass productivity between run 11 (minimum initial biomass concentration) and run 12 (maximum initial biomass concentration), but not in the corresponding μ. This result showed that, algal cells propagated faster in the lowest cell concentration when no light inhibition occurred, but that there was considerable mutual shading in the highest cell concentration that resulted in slower cell population renewal. The air flow rate in the bioreactor affected gas exchange, mixing level and the light regime of the culture medium. When the air flow rate was at a very low level,

Table 2. Coded matrix of the effects of the initial biomass concentration, gas flow rate and CO_2 concentration on the growth parameters and response values.

Run	Factor			Growth parameter		Response value				
	Initial biomass concentration (X_1)	Gas flow rate (X_2)	CO_2 concentration (X_3)	Productivity ($mg\ L^{-1}h^{-1}$)	μ (h^{-1})	Carbon content (100%)	R_C ($mg\ L^{-1}h^{-1}$)	CO_2 utilization efficiency (100%)	Starch content (100%)	P_S ($mg\ L^{-1}h^{-1}$)
1	-1.00	-1.00	-1.00	11.67	0.0138	45.32	19.40	3.29	20.96	2.45
2	-1.00	1.00	1.00	29.15	0.0299	43.81	46.83	0.88	31.83	9.28
3	1.00	-1.00	1.00	19.99	0.0072	46.49	34.07	1.93	29.11	5.82
4	1.00	1.00	-1.00	17.51	0.0066	45.84	29.44	1.67	37.26	6.53
5	0.00	0.00	0.00	29.19	0.0143	44.15	47.25	2.00	33.64	9.82
6	-1.00	-1.00	1.00	11.67	0.0130	44.32	18.96	1.07	27.19	3.17
7	-1.00	1.00	-1.00	34.17	0.0341	44.80	56.14	3.18	28.98	9.90
8	1.00	-1.00	-1.00	13.34	0.0046	44.78	21.91	3.72	30.61	4.08
9	1.00	1.00	1.00	20.00	0.0068	45.83	33.61	0.63	24.29	4.86
10	0.00	0.00	0.00	29.15	0.0149	44.23	47.27	2.01	33.59	9.79
11	-1.68	0.00	0.00	10.01	0.0545	46.19	16.96	0.72	33.35	3.34
12	1.68	0.00	0.00	14.20	0.0038	46.04	23.98	1.02	34.67	4.92
13	0.00	-1.68	0.00	10.79	0.0059	45.42	17.97	5.08	17.76	1.92
14	0.00	1.68	0.00	27.52	0.0140	45.84	46.26	1.06	30.62	8.43
15	0.00	0.00	-1.68	23.31	0.0121	45.76	39.11	10.18	30.55	7.12
16	0.00	0.00	1.68	15.85	0.0085	44.68	25.97	0.60	20.93	3.32
17	0.00	0.00	0.00	29.27	0.0149	44.33	47.57	2.02	33.93	9.93

the algae growthrate slowed down due to poor gas exchange, lower mixing levels and algal cells settling (Merchuk et al., 2007). Run 13 confirmed this. On the other hand, it was harmful to the algae growth when the air flow was at a very high level, be-cause the excessive gas flow induced a higher shear level (Brindley Alías et al., 2004). The productivity level of run 14 was slightly lower when compared with the control group, probably due to the high shear level. CO_2 is a very important carbon source of algal photosynthesis, so supplementing CO_2 in the course of algal culture can promote cell proliferation, inhibit photo respiration and enhance photosynthetic efficiency (Iwasaki et al. 1998). Run 7 and the control groups (run 5, 10, 17) showed it was beneficial to algae growth to supplement 5 to 10% (V/V) CO_2; this result is in accordance with Chiu et al. (2008) who supplemented 5% CO_2 in a *Chlorella sp.* culture and obtained higher biomass productivity and specific growth rate. The CO_2 fixation efficiency of all runs ranged from 0.60 to 10.18%, similar to the reports of Zhang et al. (2002) and Chiu et al. (2008). As there is an inverse relationship between CO_2 utilization efficiency and CO_2 concentration, the quantity of supplemental CO_2 can be decreased to increase the economical efficiency. In addition, introducing a membrane module (Cheng et al., 2006), optimizing the mixing device (Ryu et al., 2009) and improving the gas sparger (Talbot et al., 1990) also contributed to increasing the CO_2 utilization efficiency.

The effect of operational conditions on CO_2 fixation rate

To investigate the effects of cultivation conditions on CO_2 fixation rate, a five-level central composition design was introduced. As Table 2 shows, the carbon content of the algal cells ranged from 43.81 to 46.49% in different operational conditions and a t-test ($p < 0.05$) indicated that there was no evident difference between all runs, namely, initial biomass concentration, gas flow rate and CO_2

Table 3. Regression coefficients of polynomial functions for different responses.

Factor	CO_2 fixation rate					Starch content					Starch productivity				
	Effect	S.D.	t(2)	p	coefficient	Effect	S.D.	t(2)	p	coefficient	Effect	S.D.	t(2)	p	coefficient
Constant	47.02	0.10	452.11	<0.00001	47.02	33.64	0.11	318.08	0.00001	33.64	9.77	0.04	228.79	0.00002	9.77
X1(L)	-1.54	0.10	-15.75	0.00401	-0.77	2.13	0.10	21.42	0.00217	1.06	-0.12	0.04	-3.11	0.08973	-0.06
X1(Q)	-16.65	0.11	-154.89	0.00004	-8.32	0.72	0.11	6.56	0.02246	0.36	-3.50	0.04	-79.23	0.00016	-1.75
X2(L)	17.47	0.10	178.82	0.00003	8.73	5.29	0.10	53.24	0.00035	2.64	3.81	0.04	94.95	0.00011	1.90
X2(Q)	-8.41	0.11	-78.26	0.00016	-4.21	-6.23	0.11	-56.95	0.00031	-3.11	-2.76	0.04	-62.56	0.00026	-1.38
X3(L)	-2.27	0.10	-23.23	0.00185	-1.13	-3.16	0.10	-31.78	0.00099	-1.58	-0.91	0.04	-22.73	0.00193	-0.46
X3(Q)	-8.13	0.11	-75.47	0.00018	-4.06	-5.14	0.11	-46.93	0.00045	-2.57	-2.73	0.04	-61.80	0.00026	-1.37
X1X2	-14.38	0.13	-112.70	0.00008	-7.19	-2.71	0.13	-20.86	0.00229	-1.35	-3.02	0.05	-57.65	0.00030	-1.51
X1X3	6.52	0.13	51.08	0.00038	3.26	-5.89	0.13	-45.36	0.00049	-2.94	-0.01	0.05	-0.17	0.88373	-0.005
X2X3	-4.21	0.13	-33.02	0.00092	-2.11	-3.71	0.13	-28.60	0.00122	-1.86	-1.19	0.05	-22.68	0.00194	-0.59

concentration had almost no effect on cell carbon content. On the contrary, operational conditions had a strong effect on the CO_2 fixation rate, which ranged from 16.96 (run 11), to 56.14 mg L^{-1} h^{-1} (run 7). These results were higher than those obtained by Yun et al. (1997), who obtained rates of 23.4 mg L^{-1} h^{-1} for the microalga *Chlorella vulgaris* (UTEX 259) under conditions of 27°C, 15% CO_2 and 110 µE m^{-2} s^{-1}. This suggested the potential of applying *T. subcordiformis* to the carbon fixation process. In addition, as there was no evident difference in the carbon content of all runs, according Equation 6, the effect of the operation conditions on the CO_2 fixation rate was similar to those on algae productivity.

Table 3 shows the results for the effects of and the interactions between the factors of the initial biomass concentration, gas flow rate and CO_2 concentration, as well as the coefficients of the model with the CO_2 fixation rate as the response. An analysis of this table showed that in the range evaluated, the CO_2 fixation rate was controlled by the gas flow rate (L), initial biomass concentration (Q) and interaction between initial biomass concentration (L) and gas flow rate (L), in the order of importance. The other factors and interactions showed a lower proportion of statistical importance. According to Table 3, Equation 8 can be obtained, which represents the statistical model for the variable response to CO_2 fixation rate:

$$Y = 47.02 - 0.77X_1 + 8.73X_2 - 1.13X_3 - 8.32X_1^2 - 4.21X_2^2 - 4.06X_3^2 - 7.19X_1X_2 + 3.26X_1X_3 - 2.11X_2X_3 \quad (8)$$

In Figure 2, the contour curves, shows the variation in the CO_2 fixation rate as a function of the factors studied. These results reflect that an increase of CO_2 fixation rate needs appropriate operational conditions, which are fixing the initial biomass concentration at the central region, the gas flow rate in the highest region and the CO_2 concentration at the central region.

According to the statistical model and the contour curves, the CO_2 fixation rate can reach the maximum predicted value of 59.93 mg L^{-1} h^{-1} when the initial biomass concentration is 340 mg L^{-1}, the gas flow rate is 0.46 V V^{-1} m^{-1} and the CO_2 concentration is 2.9% (Table 5).

The model for the variable response to CO_2 fixation rate was validated from the F distribution (Table 4), which suggested the existence of a quadratic relationship between the variables, indicating that the proposed model fitted the experimental data well.

The effect of operational conditions on starch content

As Table 2 shows, unlike carbon content, the starch content of the algal cell was clearly affected by the culture operational conditions and it ranged from 17.76 to 37.26%. A study by Hu et al. (1998) indicated that the biomass concentration directly affected light distribution in the bioreactor, the accepted light level of the algal cell and the level of metabolized carbohydrate. It was also found that, the content of carbohydrate decreased with increasing biomass concentrations when *Chlorococcum littorale* cell density was less than 10 g L^{-1} in the bioreactor and the inverse tendency when it was more than 10 g L^{-1}. In this study, no similar phenomenon was observed, as the results of run 11 (the minimum initial biomass concentration) and run 12 (the maximum initial biomass

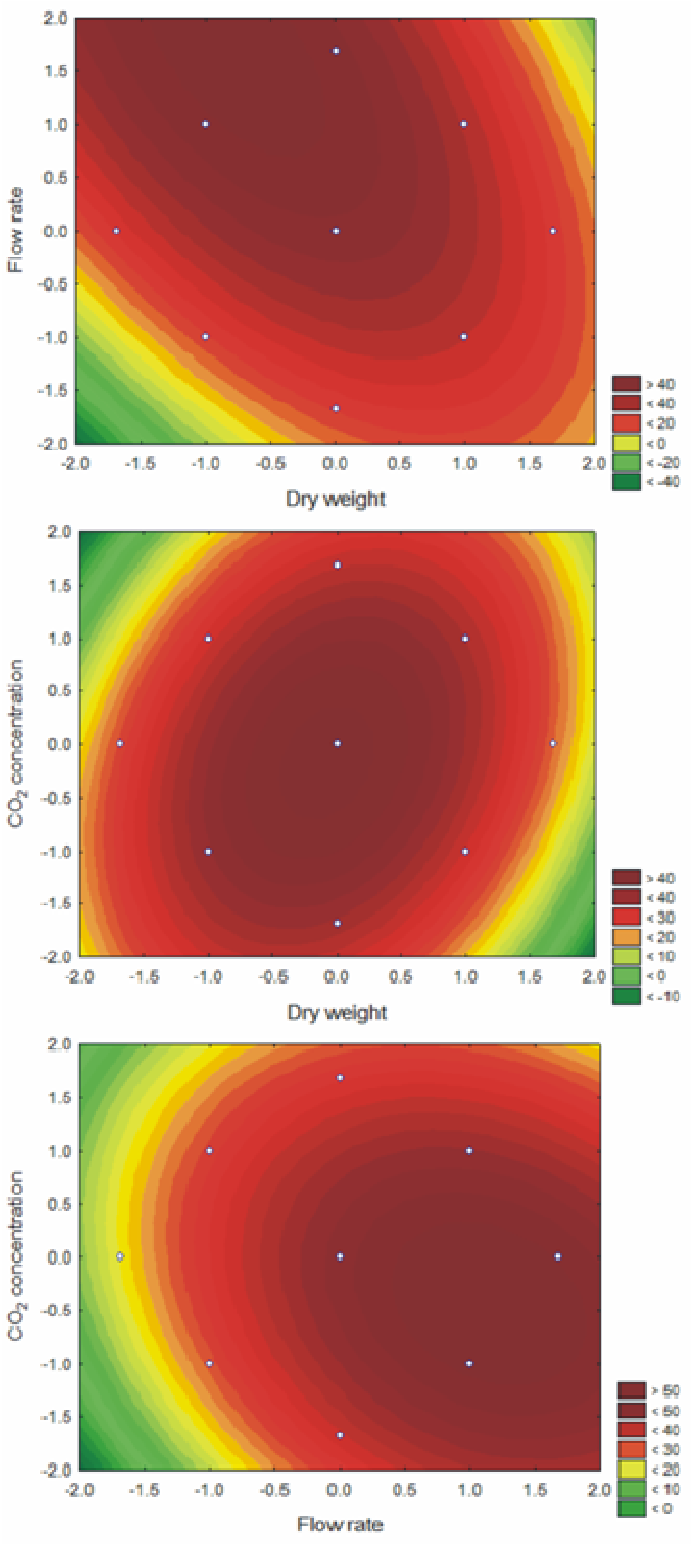

Figure 2. Contour curves for the variable CO_2 fixation rate.

concentration) were approximately equal. Thus, there was less effect of initial biomass concentration on starch content in the experimental range. The gas flow rate in the bioreactor not only affected gas-liquid transfer and mixing levels, but also affected the carbohydrate content in algal cells (Fabregas et al., 1995). As Table 2 shows,

Table 4. Analysis of variance (ANOVA) table for refined models.

Model	SS	Degrees of freedom	MS	F	P-value
CO_2 fixation rate					
Regression	2439.76	9	271.08	7.77	0.0066
Residues	244.28	7	34.90		
Total	2684.04	16			
Starch content					
Regression	435.04	9	48.34	7.35	0.0077
Residues	46.05	7	6.58		
Total	481.09	16			
Starch productivity					
Regression	122.64	9	13.63	7.66	0.0068
Residues	12.45	7	1.78		
Total	135.09	16			

Table 5. Results of DFA optimization.

Parameter	Operational condition			Response			
	Initial biomass concentration	Gas flow rate	CO_2 concentration	R_c (mg $L^{-1}h^{-1}$)	P_s (mg $L^{-1}h^{-1}$)	CO_2 utilization efficiency (%)	Biomass productivity (mg $L^{-1}h^{-1}$)
Optimal condition for R_c	-1.46	2.64	-1.41	59.93	7.49	3.71	34.11
Optimal condition for P_s	-0.46	1.02	-0.39	54.76	10.85	1.91	33.38
DFA optimization	-0.68	1.46	-0.62	57.03	10.63	2.03	34.38
DFA validated value				55.15[a]	10.66[a]		

[a] represents experimental values; the others are predicted values.

the starch content of the algal cell was comparatively low (17.76%) when the gas flow rate was low (run 13). This was due to a lesser gas flow rate given as bad gas exchange environment, which then caused starch consumption by photorespiration (Foyer and Noctor, 2000). Thus, increasing the gas flow rate favored starch accumulation, but this needs to be controlled in a reasonable range that does not result in shear stress on the algal cell. Izumo et al. (2007) reported that the CO_2 concentration in algal culture affected the content and physicochemical properties of starch in *Chlorella kessleri* 11 h. In this experiment, similar results were obtained. Run 15 and run 16 showed that the intracellular starch content decreased with increasing CO_2 concentration, which was similar to the report by de Castro Araújo and Garcia (2005). On the other hand, the quantity of exopolysaccharides, another kind of photosynthetic product, would increase in the environments of high CO_2 concentration, so that the content of intracellular starch would decrease (González López et al., 2009).

As shown in Table 3, the order of importance in

affecting starch content is the gas flow rate (Q), the interaction between the initial biomass concentration (L) and CO_2 concentration (L), the gas flow rate (L) and CO_2 concentration (Q). The other factors and interactions showed a lower proportion of statistical importance. According to Table 3, Equation 9 can be obtained, which represents the statistical model for the variable response starch content:

$$Y=33.64+1.06X_1+2.64X_2-1.58X_3+0.36X_1^2-3.11X_2^2-2.57X_3^2-1.35X_1X_2-2.94X_1X_3-1.86X_2X_3 \quad (9)$$

The contour curves (Figure 3) showed the variation in the intracellular starch content as a function of the factors studied. Thus, an increase in intracellular starch content was obtained not only by altering the CO_2 concentration to a lower level, that is, carrying out the cultivation under reduced proportions of CO_2 enrichment in the air entering the photobioreactor, but also by fixing the gas flow rate in the highest region. Unlike the two factors stated earlier, the initial cell concentration had no evident effect on the intracellular starch content.

The model for the variable response intracellular starch content was validated from the F distribution (Table 4) that suggested the existence of a quadratic relationship between the variables, indicating that the proposed model fitted the experimental data well.

The effect of operational conditions on starch productivity

As Table 2 shows, the highest starch productivity reached 9.93 mg L^{-1} h^{-1} (run 17), and the result was higher than those obtained by Maeda et al. (2006), who obtained intracellular starch productivity of 4.1 mg L^{-1} h^{-1} (calculated) for the microalga *Chlamydomonas* sp. in a turbidostat culture. According Equation 7, the rate of starch production is the result of biomass productivity and intracellular starch content. Hence, the change in starch productivity was a combined action of the biomass productivity and the intracellular starch content. When compared with the previous two responses, the optimal region of the contour curve (Figure 4) with starch productivity as the response was most inclined to the central region. This means that carrying out the cultivation under moderate, initial biomass concentrations and CO_2 atmosphere was favored and the intracellular starch productivity was increased.

As Table 3 shows, the effect of the first three terms on starch productivity in order of importance is the gas flow rate (L), the initial biomass concentration (Q), the interaction between the initial biomass concentration (L) and the gas flow rate (L). The other factors and interactions showed a less important statistical significance. According to Table 3, the statistical model for the variable response starch productivity can be obtained (Equation 10):

$$Y=9.77-0.06X_1+1.90X_2-0.46X_3-1.75X_1^2-1.38X_2^2-1.37X_3^2-1.51X_1X_2-0.005X_1X_3-0.59X_2X_3 \quad (10)$$

According to the statistical model and the contour curves, starch productivity can reach the maximum predicted value of 10.85 mg L^{-1} h^{-1} when the initial biomass concentration is 1340 mg L^{-1}, the gas flow rate is 0.30 V V^{-1} m^{-1} and the CO_2 concentration is 8.1% (Table 5).

The model for the variable response starch productivity was validated from the F distribution (Table 4), which suggested the existence of a quadratic relationship between the variables, indicating that the proposed model fitted the experimental data well.

Simultaneous optimization of two responses

In the above results, the maximal CO_2 fixation rate and starch productivity were obtained in different optimal regions. However, in this study, the objective was given a proper compromise region in which the CO_2 fixation rate and starch productivity were at a satisfactory level. Taking this into consideration, a DFA was used to find the best compromise between the two responses based on the mathematical models constructed in RSM.

The responses, CO_2 fixation rate (Y_1) and starch productivity (Y_2), were transformed into the corresponding desirability scales d_1 and d_2, and D was obtained according to Equations 3 and 4. During this step, it should be noted that stringency can be added to a chosen predicted optimum by increasing or decreasing the Y^*_i and Y_i^* used during optimization. In this study, with the goal of choosing the optimal operational conditions for both CO_2 fixation rate and starch productivity simultaneously, it proved sufficient to use Y^*_i equal to the highest and Y_i^* equal to the lowest observed value in the experimental range, respectively.

The contour curves (Figure 5) show the overall desirability D as a function of the factors studied. The visible similarity of Figures 4 and 5 indicate that the DFA optimized region was close to the optimal region for P_s and was relatively removed from the optimal region for R_c. As a result, the predicted values of responses in the optimal region for P_s and the DFA-optimized region had no evident difference (Table 5) except a slightly higher CO_2 fixation rate. From the angle of CO_2 utilization efficiency and biomass productivity, the DFA-optimized region was advisable. On the other hand, when compared with the optimal region for R_c, the DFA-predicted P_s reached 10.63 mg L^{-1} h^{-1}, which was much higher than that of the optimal region for R_c (7.49 mg L^{-1} h^{-1}). This meant that starch productivity was more sensitive than the CO_2 fixation rate for changing operational conditions.

Optimal culture conditions validation

As from the DFA optimization (Table 5), which suggested

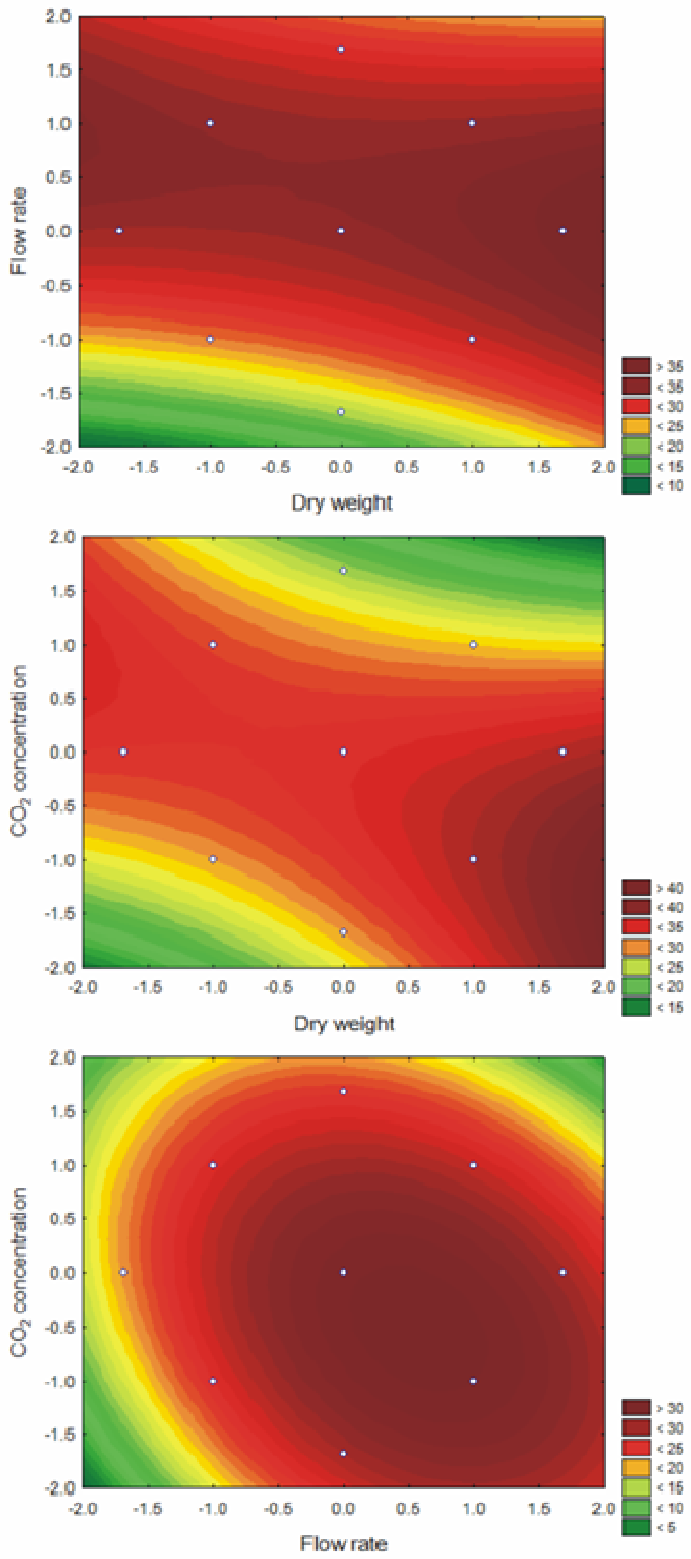

Figure 3. Contour curves for the variable starch content.

maintaining the initial biomass concentration at 1120 mg L^{-1}, fixing gas flow rate at 0.35 V V^{-1} m^{-1} and injecting air enriched with 6.9% CO_2, cultures were carried out under these conditions in order to prove its validity. The results showed that the predicted value of DFA optimization fitted the experimental data well. The validated value

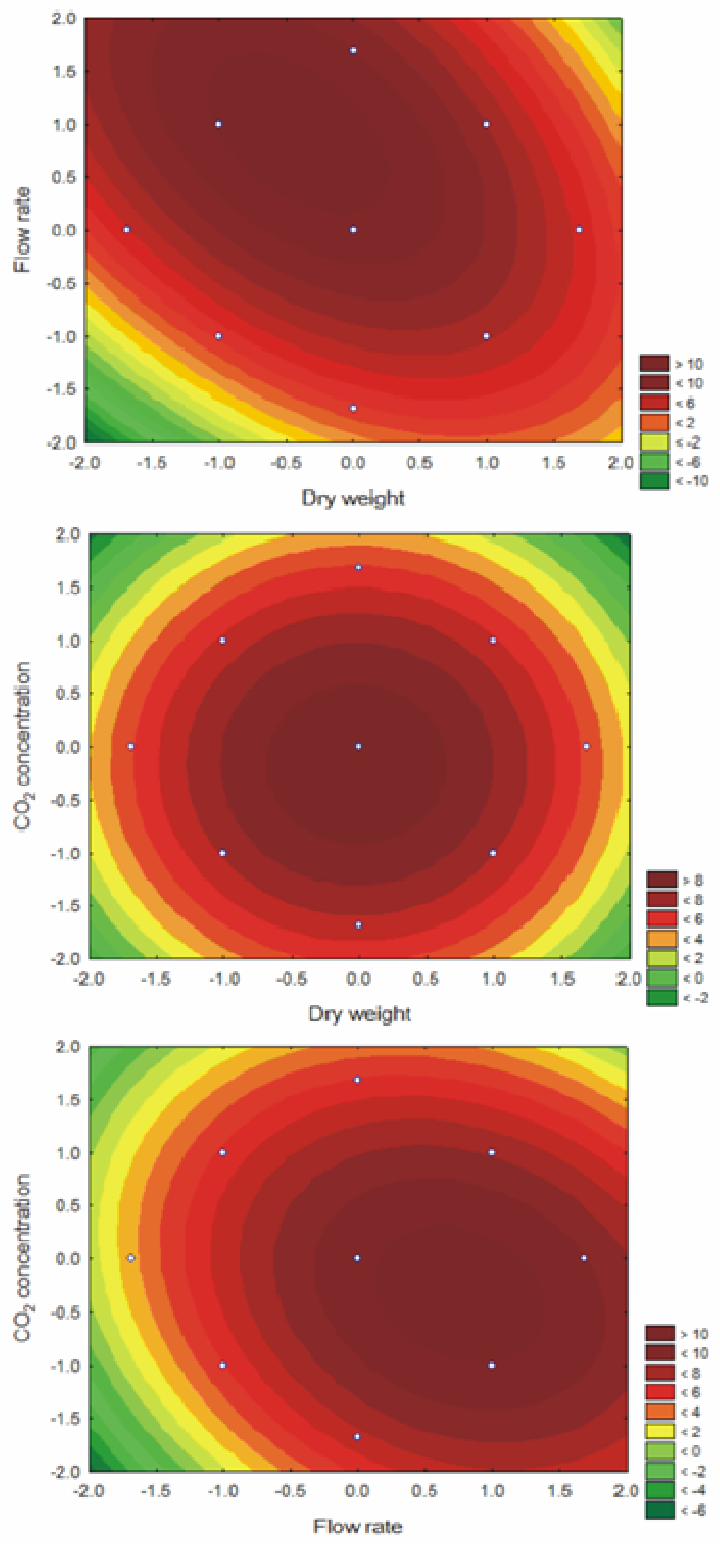

Figure 4. Contour curves for the variable starch productivity.

of R_c (55.15 mg $L^{-1}h^{-1}$) was almost invariable and the value of P_s (10.66 mg $L^{-1}h^{-1}$), which increased to 7.4% when compared with the maximum values of previous optimized experiments (Table 2). It was concluded that the optimization of DFA can present compromises at which the CO_2 fixation rate and starch productivity can be in

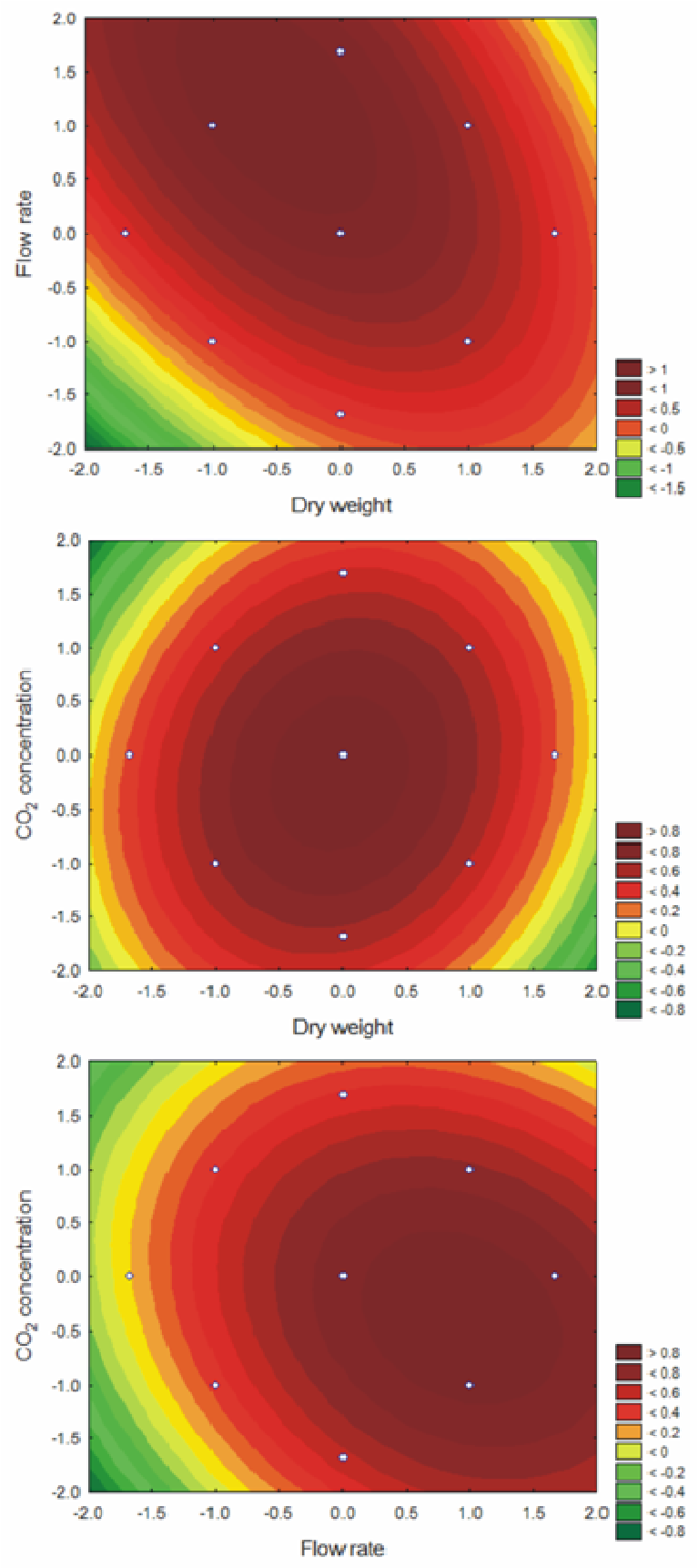

Figure 5. Contour curves for the DFA optimization.

desired levels.

Conclusions

The initial biomass concentration in the bioreactor, the gas flow rate and the CO_2 concentration in the culture were determining factors in the carbon fixation and starch accumulation processes of the marine microalga *T. subcordiform*. During the course of optimization, RSM was adequate for determining the effects of the factors and the processes were fitted to a model. By the optimization of the combined RSM and DFA, the CO_2 fixation rate and starch productivity was obtained at the levels of 55.15 mg L^{-1} h^{-1} and 10.66 mg L^{-1} h^{-1}, respectively, under operational conditions of maintaining the initial biomass concentration at 1120 mg L^{-1}, fixing the gas flow rate at 0.35 V V^{-1} m^{-1} and injecting air enriched with 6.9% CO_2. These results suggest the potential for applying *T.subcordiform* to CO_2 mitigation and starch production.

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