Optimization of nutrient stress using *C. pyrenoidosa* for lipid and biodiesel production in integration with remediation in dairy industry wastewater using response surface methodology

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Abstract

The present study illustrates optimization and synergetic potential of alga *Chlorella pyrenoidosa* for lipid production and remediation of Dairy industry wastewater (DIWW) through response surface methodology (RSM). Maximum lipid productivity of 34.41% was obtained under 50% DIWW supplemented with 0 mg L⁻¹ nitrate (NO₃⁻), and 50 mg L⁻¹ phosphate (PO₄⁻³). While maximum biomass productivity (1.54 g L⁻¹) was obtained with 50% DIWW supplemented with 100 mg L⁻¹ NO₃⁻, and 50 mg L⁻¹, PO₄⁻³. Maximum removal of COD (43.47%), NO₃⁻ (99.80%) and PO₄⁻³ (98.24%) was achieved with 8th run (75% DIWW, 150 mg L⁻¹ NO₃⁻, 75 mg L⁻¹ PO₄⁻³), 15th run (50% DIWW, 0 mg L⁻¹ NO₃⁻, 50 mg L⁻¹, PO₄⁻³) followed by 1st run (25% DIWW, 50 mg L⁻¹ NO₃⁻, and 25 mg L⁻¹, PO₄⁻³), respectively. Lipid (bio-oil) obtained from 15th run of experiment was converted in biodiesel through base catalyze transesterification process. Fatty acid methyl ester (FAME) analysis of biodiesel confirmed the presence of major fatty acids in *C. pyrenoidosa* grown in DIWW were C11:0, C14:0, C16:0, C16:1, C18:1 and C18:2. Results of study clearly demonstrate enhanced growth and lipid accumulation by *C. pyrenoidosa* in surplus PO₄⁻³ and limitation of NO₃⁻ sources with DIWW and its suitability as potential alternative for commercial utilization.

Keywords Algal biomass · Wastewater · Nutrients · Lipid · Biodiesel · Response surface methodology

Introduction

Crude oil is the world's largest fossil fuel source to meet the primary energy demand, satisfying 31.3% of the world's total energy demand of 13,699 MT. This may critically explain why the total global emission of CO₂ increased from 15,458 to 32,381 MT during 1973 and 2014 (Lithgow 2017). So, excessive use of fossil fuel for energy generation has proven to be unsustainable from an environmental and economic point of view. Another major environmental challenge is linked to the management of wastewater discharge from industrial sectors such as dairy, textiles and agro-processing (Gothwal et al. 2012). Kushwaha et al. (2011) estimated that Indian dairy industries generate about 275 MT of wastewater annually. In European countries, the average effluent discharge from dairy industry is about 500 m³ day⁻¹ (Kothari et al. 2013). Dairy effluent is characterized by high turbidity and pungent smell, high biochemical oxygen demand (BOD) (40–48,000 mg L⁻¹) and chemical oxygen demand (COD) (80–95,000 mg L⁻¹), and variable pH (6–9) (Demirel et al. 2005). The conventional techniques (physical and chemical)

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available for the treatment of DIWW include oil-water separator, grease removal, flotation of suspended solids, equalization of flow and clarifiers to eliminate the suspended solids. But these methods are inappropriate as well as energy intensive, which results in unsustainable wastewater treatment (Shete and Shinker 2013).

Kothari et al. (2012) reported that the twin challenge of energy demand and wastewater treatment can be achieved in a more sustainable manner using an integrated approach within a cost effective manner. In this regard, microalgae have enormous potential to generate biomass and accumulate lipid (20–50% dry cell weight) under varying culture conditions such as lab cultivation, freshwater streams, marine water and wastewater (Kothari et al. 2017). Cultivation of algae on industrial wastewater not only reduces the water foot print of algal cultivation but also helps in energy recovery through biodiesel production. Researchers across the world have realized that biofuel generated from biomass has potential to reduce the dependency on conventional fossil fuels and biofuel represents a potential fuel of the future (Singh et al. 2014a, b; Kothari et al. 2017). Previous studies supported that the algal based biofuel production using wastewater holds potential to meet the energy requirements. This integrated approach has proven to be a more suitable way for sustainable wastewater treatment (Wang et al. 2010).

Wastewater from dairy industry contains high nutrient load that can be utilized for the growth of a variety of algal strains (Hena et al. 2015). Growing algal species establish a synergistic relationship with aerobic bacteria present in wastewater and lead to the degradation of organic matter present in the water (Pathak et al. 2014). *Chlorella pyrenoidosa* is a fast growing (doubling⁻¹) microalga, which is well studied for the treatment of industrial wastewater and biodiesel production. Several investigations have highlighted the potential of *C. pyrenoidosa* for its efficient potential to treat the wastewater with increased lipid production. Interestingly, Wang et al. (2010) reported the enhancement in fatty acid content (9–13%) in *Chlorella* sp. cultivated in digested dairy manure. They also observed that *Chlorella* sp. removed ammonia, total nitrogen (TN), total phosphorus (TP) and chemical oxygen demand (COD) by 100, 82.5, 74.7 and 38.4%, respectively. Chu et al. (2015) estimated the efficiency of *C. pyrenoidosa* for sustainable biofuel production based on their lipid productivity and treatment efficiency using starch wastewater, and found high biomass concentration (1.29–1.69 g L⁻¹) and lipid productivity (43.37 mg L⁻¹ day⁻¹) with a reduction in TN and TP about 78.7 and 97.2%, respectively. Recently, Chokshi et al. (2016) studied that dairy industry wastewater induced 5–6 times more lipid production in *Acutodesmus dimorphus* than the BG-11 nutrient medium (149 mg L⁻¹) over 8 day cultivation period. The phenotypic plasticity of alga allows them to grow under stressful conditions which results enhanced lipid

accumulation (Yu et al. 2015). Singh et al. (2014) suggested that culture conditions of alga should be optimized by varying the combinations of key variables such as pH, nutrients (N and P) and temperature to maximize lipid production. An optimized concentration of nitrogen, for example, can establish a metabolic balance between the rate of carbon fixation and rate of nitrogen assimilation (Adams et al. 2013). Whilst the application of response surface methodology (RSM) for multivariate optimization of drug dosages has been well studied (Azma et al. 2011; Kirrolia et al. 2014), but studies on the use of RSM for the optimization of culture conditions for algal lipid synthesis are very less. RSM is a statistical methods for modeling analysis and prediction of experimental findings in which the response of interest is evaluated by different variables with objectives of optimizing this response (Montgomery 2017; Karemore et al. 2013). There are very few research studies on the application of RSM available for optimization of the integrated treatment approach of DIWW for lipid production using microalgae. Therefore, present study focuses on identifying the optimum nutrient concentration for biodiesel production and pollution reduction from DIWW through RSM using *C. pyrenoidosa* simultaneously.

Materials and methods

Collection of algal strain and culture conditions

Chlorella pyrenoidosa (NCIM 2738) was obtained from the National Collection of Industrial Microorganisms (NCIM, Pune) and cultured in BG-11 medium (in g L⁻¹): NaNO₃, 1.5; K₂HPO₄, 0.04; MgSO₄·7H₂O, 0.075; CaCl₂·2H₂O, 0.036; citric acid, 0.006; ferric ammonium citrate, 0.006; EDTA (disodium salt), 0.001; Na₂CO₃, 0.02; 1 mL trace elements solution (in g L⁻¹: H₃BO₃, 2.86; MnCl₂·4H₂O, 1.81; ZnSO₄·7H₂O, 0.222; NaMoO₄·2H₂O 0.39; CuSO₄·5H₂O, 0.079; Co(NO₃)₂·6H₂O, 0.0494), with a pH of 7.0 ± 1. Media and flasks were sterilized by autoclaving for 20 min at 15 Psi and 121 °C before use. All glasswares were cleaned and sterilized at 120 °C for 6–7 h before use. The medium was inoculated with culture density equivalent to 0.1 at 660 nm and incubated at 25 ± 1 °C in a 12 h light (10 W m⁻²)/dark cycle.

Collection of dairy industry wastewater (DIWW)

Dairy industry wastewater (pH 7.2 ± 0.3; Total dissolved solids, 975 ± 12.5 mg L⁻¹, chloride, 310 ± 6.5 mg L⁻¹, hardness, 471.5 ± 7 mg L⁻¹; nitrate, 58.5 ± 3.5 mg L⁻¹; phosphate 18.6 ± 2.4 mg L⁻¹; alkalinity 983.5 ± 5 mg CaCO₃ mg L⁻¹, BOD, 618.75 ± 1.5 mg L⁻¹, COD, 1074.35 ± 11.2 mg L⁻¹) was collected from Producer Cooperative Milk Union Limited,

Lucknow (Uttar-Pradesh, India) which has the capacity to produce an average of 1MLD of wastewater per day. The wastewater samples were collected in sterilized sampling bottles and stored at 4 °C before use. The characterization of wastewater was performed according to Standard Methods for the Examination of Water and Wastewater (2012).

Experimental design for optimization of culture conditions and data analysis

In conventional optimization processes, an individual factor is varied while keeping the remaining factors constant, which represents a single factor time operation. Conventional experimental and statistical methods are, therefore, unable to explain the effects of the interaction of variables. Thus, RSM provides a systematic statistical design approach to explore the combined influence of two or more variables used in the experiment. In the present study, *C. pyrenoidosa* was grown under various manipulated concentrations of nitrate (0, 50, 75, 100, 200 mg L⁻¹), phosphate (0, 25, 50, 75, 100 mg L⁻¹) and DIWW (0, 25, 50, 75, 100%). Biomass productivity (g L⁻¹), lipid content (%) and percentage removal of pollutant load (COD, NO₃⁻ and PO₄⁻³) were considered as response variables. A central composite design (CCD) of RSM was employed using Design Expert software-10 (Student version). CCD is full-factorial two-level design which is a mathematical and statistical method which can be operated in different optimization parameters. CCD optimizes the process as well as reduces time and cost in experiment (Bala et al. 2016). The use of RSM-CCD recognizes optimized condition of multiple variables in single set of experimental combination.

The experimental design consisted of three nutrient stress variables: NaNO₃ (nitrogen source), K₂HPO₄ (phosphorus source) and DIWW (pollutant stress and aquatic medium). All experiments were performed in 500 mL conical flasks with light intensity of 10 w/m² using cool fluorescent tubes in a culture room at temperature of 25 ± 1 °C. Table 1 indicates the five levels of variables for selected factors. A total of 20 experiments containing varying nutrient concentrations were performed. The effect of independent factor on dependent variable was analyzed by a polynomial Eq. (1) expressed as:

$$Y = B_0 + \sum_{i=1}^k B_i + \sum_{i=1}^k B_{ii}x_i^2 + \sum_{i=1}^{k-1} \sum_{j=2}^k B_{ij}x_i x_j + e_{ij}, \quad (1)$$

where Y is the response of prediction and $x_i, x_j, \dots, x_k$ are the input variables, which affect the response Y , $x_i^2, x_j^2, \dots, x_k^2$ are the square effect of variables; $x_i x_j, x_i x_k, \dots, x_j x_k$ are the interaction effect of variables, B_0 is the intercept; B_i ($i = 1, 2, \dots$) is the linear effect; B_{ii} ($i = 1, 2, \dots$) is the effect of squared, B_{ij} ($i = 1, 2, \dots, k, j = 1, 2, \dots$) is the effect of interaction; and random error is denoted by e .

Measurements of algal growth

Microalgae growth was measured by observing the optical density (O.D.) using UV-Vis spectrophotometer (Shimadzu Model, Japan) at 680 nm. All cultures were inoculated with initial O.D. of 0.1 cell were concentrated and dried (60 °C) and expressed in terms of g L⁻¹. From the calibration curve observed from Optical density at 680 nm and dry algal biomass, was found to be equivalent to 0.61 g L⁻¹ of biomass. Therefore, biomass concentration was calculated by the regression equation $y = 0.613x$ ($R^2 = 0.98$), where y is the dry cell weight (g L⁻¹) and x is the absorbance at 680 nm.

Kinetics of algal growth

A growth model was applied to measure the kinetics of microalgal growth. Specific growth rate was calculated using Eq. (2),

$$\mu = \ln(N_o - N_t)/(t_1 - t_0). \quad (2)$$

Here, μ is the specific growth rate of *C. pyrenoidosa* (mg L⁻¹ day⁻¹), calculated using the Graph Pad Prism Software v6 (student version) by applying the commonly used exponential Eq. (3),

$$N_t = N_o \exp(r \times t) \quad (3)$$

In Eqs. (2 and 3), N_t is the size of the population at time t and N_o is the initial population size; r is the population growth rate and t represents the days of our experiment (Van et al. 2015) until all treatments had approximately reached their maximum densities (15 days) were used for calculation.

Extraction of lipid (bio-oil)

Lipids were extracted from the biomass obtained from each experimental set-up by following the Modified Bligh and Dyer (MBD) method (1959) using chloroform and methanol

Table 1 Code limit for variables used in the experimental design

Factor	Unit	Symbol	Coded level				
			−α	−1	0	+1	+α
DIWW	%	X_1	0	25	50	75	100
NO ₃ ⁻	mg L ⁻¹	X_2	0	50	100	150	200
PO ₄ ⁻³	mg L ⁻¹	X_3	0	25	50	75	100

in 2:1(v/v) ratio (Wacker et al. 2016). The lipid content in algal biomass was measured by following the Eq. (4) as given below:

$$\text{Lipid content (\%)} = \frac{\text{lipid content (mg)}}{\text{algal biomass (g)}} \times 100. \quad (4)$$

Nutrient removal (COD TN, and TP)

For quality analysis of wastewater, cultures of *C. pyrenoidosa* were centrifuged (5000 rpm, 10 min at 4 °C), and the supernatant was subsequently filtered through a 0.45 mm filter paper. The filtrate was analyzed for COD, NO_3^- and PO_4^{3-} . All analyses were carried out according to standard methods (Standard Methods for the Examination of Water and Wastewater 2012), after which removal efficiency of nutrient was calculated using Eq. (5)

$$\text{Nutrient removal (\%)} = (S_{\text{po}} - S_{\text{pt}}/\Delta t) \times 100, \quad (5)$$

where S_{po} (mg L^{-1}) is initial concentration and S_{pt} (mg L^{-1}) final concentrations of process variables (COD, TN, and TP) after analysis.

Transesterification of algal oil and fatty acid methyl ester (FAME) analysis

A mixture of catalyst (NaOH) and methanol was poured into the flask containing the algal oil, which was then maintained for 3 h on a continuous rotator shaker (200 rpm) to allow the reaction for completion. After 3 h, the biodiesel formed in the upper layer and the pigment along with glycerine settled at the bottom. Biodiesel was separated with the help of a separating funnel (Hossain et al. 2008). Gas chromatography (GC) equipped with flame ionization detector (FID) (Perkin Elmer, USA) was used to analyze the composition of methyl esters produced from algal oil. The FAME analysis was carried out with a split injection onto an analytical column with a polar stationary phase and a flame ionized detector (FID).

Statistical analysis using RSM

Analysis of variance (ANOVA) and 't' test was performed on the experimental data obtained in present study using Design Expert software-10 (student version).

Results and discussion

Influence of DIWW with manipulated nutrient concentration on algal growth kinetics

Data shown in Fig. 1 revealed that algal growth pattern influenced by culture medium conditions. Data for variables of

Fig. 1 Growth kinetics of *C. pyrenoidosa* grown at different concentration of DIWW, NO_3^- and PO_4^{3-}

growth kinetics such as O.D. (a), rate constant (K) and doubling time (μ) are provided in Table 2. In a total of 20 experimental runs, the highest rate constant value (0.65 day^{-1}) for algal growth was obtained with 19th run, which was designed with combination of 50% DIWW, 100 mg L^{-1} , NO_3^- and 50 mg L^{-1} , PO_4^{3-} concentrations. Similarly, maximum biomass concentration in terms of O.D was also obtained in the 19th run. The minimum algal growth rate and biomass concentration occurred in the 4th run, which was designed with a combination of 75% DIWW, 150 mg L^{-1} NO_3^- and 25 mg L^{-1} PO_4^{3-} concentrations. Thus, it is clear that phosphorus acts as a limiting factor in growth of algal biomass in comparison to nitrate and DIWW content. The increase in DIWW content was found to negatively affect algal growth, which is possibly due to excessive nutrient inhibition of algal growth. Similar findings were also observed on limited increment in growth of *Chlorella* sp. with increasing concentrations of industrial wastewater (Benavente-Valdés et al. 2016; Pathak et al. 2015).

The results suggested that the model used was highly suitable with a minimal error as shown in Fig. 2 at different concentrations of DIWW, with normal and depleted nutrient values. The stress conditions assessed such as a decrease in nutrient content appeared to accelerate neutral lipid accumulation in algal biomass (Duong et al. 2015). Previous studies showed increase in lipid accumulation leads to a decrease in growth rate but increased production of oil (James and Boriah 2010).

RSM for optimization of process variables

The Central composite design (CCD) of the RSM was proposed for identifying the optimum levels for selected

Table 2 Summary of growth kinetics for *C. pyrenoidosa* grown at different concentration of DIWW, NO_3^- and PO_4^{3-}

S.N	DIWW	Nitrate (mg L^{-1})	Phosphate (mg L^{-1})	Biomass growth (g L^{-1}) ^a	K (rate constant, day^{-1})	μ (Doubling time)
1	−1	−1	−1	0.58	0.62	2.80
2	1	−1	−1	0.46	0.61	3.36
3	−1	1	−1	0.35	0.50	3.67
4	1	1	−1	0.32	0.45	3.83
5	−1	−1	1	0.33	0.50	4.06
6	1	−1	1	1.5	0.63	1.77
7	−1	1	1	1.5	0.61	1.76
8	1	1	1	1.1	0.56	1.92
9 ^a	0	0	0	1.1	0.55	2.05
10 ^a	0	0	0	1.5	0.63	1.76
11 ^a	0	0	0	1.41	0.58	1.74
12 ^a	0	0	0	1.4	0.59	1.86
13	−1.68	0	0	1.0	0.51	2.34
14	1.68	0	0	1.54	0.57	1.69
15	0	−1.68	0	1.82	0.58	1.63
16	0	1.68	0	1.46	0.56	1.92
17	0	0	−1.68	1.53	0.57	1.89
18	0	0	1.68	0.84	0.62	2.77
19 ^a	0	0	0	1.36	0.65	2.09
20 ^a	0	0	0	1.1	0.56	2.72

^aIndicates the data points at central axis **Fig. 2** Three dimensional plot of standard error (SE) of $\text{COD}_{(\text{Removal}\%)}$ as a function DIWW, NO_3^- and PO_4^{3-} concentration

variables such as DIWW (0–100%), NO_3^- (0–200 mg L^{-1}), and PO_4^{3-} (0–100 mg L^{-1}). The second order polynomial Eq. (5) was applied to determine the relationship between variables and responses and regression coefficients were

calculated. A flat surface on the three-dimensional (3D) response graphs indicates an optimum condition for a given response, shown in Fig. 2. Analysis of variance (ANOVA) also ensures the good fit of model for biomass productivity and lipid content (Table 3). A $\text{prob} > F$ value of < 0.5 and > 0.5 , indicated the model terms which were significant and non-significant, respectively. In this assumption, the residual graph was applied to check the homogeneous effrontery by plotting the studentized residuals against the predicted value of probability. Homogeneously spreading of COD removal, TN (%), TP (%) biomass productivity and lipid content data on either side of the zero line indicated the suitability of the model in this study see in Fig. 3a–e. The adequate precision is 13.13, which measure signal-to-noise ratio, used to compare the ranges of the predicted value at design point for average predicted value with in the acceptable range, because precision value > 4 is satisfied and could, therefore, can be applied to navigate the space design in CCD. The coefficient of variance and standard deviation (SD) indicates the very high degree of accuracy and excellent reliability of the experimental values for biomass productivity (g L^{-1}) and lipid content (%) as shown in Table 4 and COD removal (%), TN (%), TP(%) in Table 5.

Table 3 The minimum central and maximum variable levels in the central composite design(CCD) for various responses (observed and predicted values) for study on *C. pyrenoidosa* grown at different concentration of DIWW, NO₃⁻ and PO₄⁻³

S.N	Factor	Observed value					Predicted value							
		DIWW (%)	NO ₃ ⁻ (mg L ⁻¹)	PO ₄ ⁻³ (mg L ⁻¹)	Biomass productivity (g L ⁻¹)	Lipid (%)	COD _(Removal) (%)	TP (%)	TN (%)	Biomass productivity (g L ⁻¹)	Lipid content (%)	COD _(Removal %)	TP (%)	TN (%)
1	-1	-1	-1	-1	0.86	29.65	42.28	99.50	98.24	0.99	30.04	41.71	99.44	95.76
2	1	-1	-1	-1	1.12	27.56	42.33	99.00	95.32	1.16	27.51	42.05	98.47	91.56
3	-1	1	1	-1	1.07	26.54	41.26	98.25	91.37	1.32	25.60	40.89	97.18	93.12
4	1	1	1	-1	0.85	25.31	42.25	98.64	69.23	1.28	25.06	41.77	99.17	70.6
5	-1	-1	-1	1	0.85	32.54	41.68	> 99	92.34	0.68	31.71	41.22	99.07	88.02
6	1	-1	-1	1	1.15	28.31	43.32	97.32	97.34	1.06	28.17	42.75	98.0	92.66
7	-1	1	1	1	1.49	24.76	41.68	97.25	85.67	1.43	23.73	41.03	97.37	86.5
8	1	1	1	1	1.36	23.65	43.47	99.60	73.25	1.61	22.18	43.10	99.26	72.82
9 ^a	0	0	0	0	1.24	28.65	42.88	> 99	96.33	0.85	28.50	42.75	99.52	95.93
10 ^a	0	0	0	0	1.54	28.65	42.88	99.00	96.33	0.85	28.50	42.75	99.52	95.93
11 ^a	0	0	0	0	0.85	28.65	42.88	99.50	96.33	0.85	28.50	42.75	99.52	95.93
12 ^a	0	0	0	0	0.62	28.65	42.88	99.40	96.33	0.85	28.50	42.75	99.52	95.93
13	-1.68	0	0	0	0.85	25.32	39.80	96.25	86.34	1.25	26.05	40.43	97.15	86.985
14	1.68	0	0	0	1.13	22.19	42.05	98.36	69.36	1.54	22.62	42.45	97.93	71.96
15	0	-1.68	0	0	1.26	34.41	42.34	99.80	93.64	0.62	34.08	42.85	> 99	> 99
16	0	1.68	0	0	1.52	23.82	41.94	99.00	85.92	1.35	25.31	42.46	99.21	81.94
17	0	0	0	-1.68	1.37	27.21	40.75	98.20	92.34	1.13	27.02	41.16	98.62	92.29
18	0	0	0	1.68	0.85	24.65	41.25	98.35	84.42	1.14	26.013	41.86	98.38	87.65
19 ^a	0	0	0	0	0.58	28.65	42.88	99.90	96.33	0.85	28.50	42.75	99.52	95.93
20 ^a	0	0	0	0	1.12	28.65	42.88	99.60	96.33	0.85	28.50	42.75	99.52	95.93

^aCentral axis

Fig. 3 Normal plot of actual versus predicted probability: **a** COD_(Removal %); **b** phosphate (%) removal; **c** Nitrate (%) removal; **d** biomass productivity (g L⁻¹); **e** Lipid content (%) associated with for *C. pyrenoidosa* grown at different concentrations of DIWW, NO₃⁻ and PO₄⁻³

Table 4 Summary of analysis of variance (ANOVA) for biomass (g L^{-1}) and lipid productivity (%) for *C. pyrenoidosa* grown at different concentration of DIWW, NO_3^- and PO_4^{3-}

Source	df	Response (1): biomass productivity (g L^{-1}); ANOVA for response surface quadric model (RSQM)				Response (2): Lipid Productivity (mg L^{-1}); ANOVA for response surface quadric model (RSQM)			
		Sum of squares	Mean square	F value	p value Prob > F	Sum of squares	Mean square	F value	p value Prob > F
Block		3.1×10^{-5}	3.1×10^{-5}			3.72	3.72		
Model	1	1.52	0.17	37.45	<0.0001*	158.35	17.59	23.28	<0.0001*
X_1	9	0.10	0.10	22.57	0.0010*	14.20	14.20	18.79	0.0019*
X_2	1	0.66	0.66	145.09	<0.0001	92.85	92.85	122.88	<0.0001*
X_3	1	6.67×10^{-6}	6.67×10^{-6}	1.47×10^{-3}	0.9702	1.23	1.23	1.63	0.2332
X_1X_2	1	0.020	0.020	4.45	0.0641	1.98	1.98	2.62	0.1400
X_1X_3	1	0.022	0.022	4.81	0.0559	0.51	0.51	0.67	0.4325
X_2X_3	1	0.089	0.089	19.76	0.0016*	6.27	6.27	8.29	0.0182*
X_1^2	1	0.53	0.53	118.21	<0.0001*	31.16	31.16	41.24	0.0001*
X_2^2	1	0.031	0.031	6.96	0.0270*	2.59	2.59	3.42	0.0972
X_3^2	1	0.14	0.14	31.72	0.0003*	7.10	7.10	9.39	0.0135*

*Values of 'Prob > F' less than 0.05 indicate model terms; NA implies that the co-efficient are not applicable for linear model

Effect of optimized process variables on remediation of pollutants from wastewater

To achieve high removal efficiency of pollutant (COD, TN and TP), the major factor NO_3^- and PO_4^{3-} using CCD model using 20 run discussed below:

COD (removal %)

Statistical analysis of the positive linear coefficient indicated that time was the most important factor affecting all responses in Eq. (6). Therefore, the relationship of COD (removal %) with variables as DIWW, TN and TP concentration using *C. pyrenoidosa* can be inferred from Eq. (6). The significant 3D plot is shown in Fig. 4 define sensitivity of all the responses with two interacting variables by accommodating the other variables at the central values. The positive value for nitrate and phosphate variable indicated that among the selected variables, these two parameters had the most pronounced effect on COD removal. The high value of 0.60 for the linear coefficient of DIWW concentration and linear (X_1), interactive (X_1X_3) and Quadric (X_1^2 & X_3^2) in Eq. (6), illustrate the significant positive effect of these variables on COD (removal%). The negative quadric effect on the coefficient of the independent variables (X_2^2) on COD (removal %) was observed as a function of these variables by keeping all other variables at a fixed level. These results suggested that a higher level of both these factors yielded a smaller response. The non-significant value (> 0.05) for all the quadric variables indicated that there was no interaction among all the variables, i.e. a change in one variable did not affect another. The COD (removal %) properties of *C. pyrenoidosa*

under different initial NO_3^- and PO_4^{3-} levels are shown in Fig. 4. The F value of 13.35 of the model was significant ($p=0.0001$), which further substantiates the suitability of the model for the given data. From the analysis by the model (R_{adj}^2 and R_{pred}^2), the R_{pred}^2 of 0.59 was not in agreement with the R_{adj}^2 of 0.83.

$$Y_{\text{COD (removal \%)}} = 42.76 + 0.6X_1 - 0.12X_2 + 0.21X_3 + 0.14X_1X_2 + 0.3X_1X_3 + 0.16X_2X_3 - 0.46X_1^2 - 0.035X_2^2 - 0.44X_3^2 \quad (6)$$

TN and TP removal

Coupling of the algal growth and lipid production with DIWW treatment implies that utilization of nutrients (TN and TP) should be examined in addition with lipid accumulation. The potential of nutrient removal (TN and TP) after 15 days of culture are shown in Table 3. The removal efficiency of phosphorus was >99%, however, the efficient removal of nitrogen was under the influence of the initial concentration of phosphorus. The PO_4^{3-} removal potential of *C. pyrenoidosa* under different NO_3^- and DIWW levels are shown in Fig. 5 which indicate and show the interactive (X_1X_2) and quadric (X_1^2) model are significant using Eq. (7) (F value of 6.21).

$$Y_{(\text{TP \%})} = 99.52 + 0.23X_1 - 0.25X_2 - 0.07X_3 + 0.74X_1X_2 - 0.027X_1X_3 + 0.14X_2X_3 - 0.70X_1^2 + 0.038X_2^2 - 0.36X_3^2 \quad (7)$$

Table 5 Analysis of variance (ANOVA) for nutrient removal, including COD, for *C. pyrenoidosa* grown at different concentration of DIWW, NO_3^- and PO_4^{3-}

Source	df	Response (3) for total phosphorus (%) ANOVA for Response Surface Quadratic Model (RSQM)			Response (3) for total nitrogen (%) ANOVA for Response Surface Quadratic Model (RSQM)			Response (3) for COD _(removal) (mg L ⁻¹) ANOVA for Response Surface Quadratic Model (RSQM)		
		Sum of squares	Mean square	F value	p value	Prob > F	Sum of squares	Mean square	F value	Prob > F
Block	1	0.36	0.36				2.69	2.69		
Model	9	14.77	1.64	3.05	0.0560		12.19	1.35	11.22	0.0007*
X_1	1	0.71	0.71	1.32	0.2810*		4.97	4.97	41.15	0.0001*
X_2	1	0.86	0.86	1.60	0.2380		0.19	0.19	1.58	0.2401
X_3	1	0.069	0.069	0.13	0.7293		0.60	0.60	4.96	0.0530*
X_1X_2	1	4.38	4.38	8.14	0.0190*		0.15	0.15	1.22	0.2973
X_1X_3	1	6.05×10^{-3}	6.05×10^{-3}	0.011	0.9179		0.71	0.71	5.85	0.0387*
X_2X_3	1	0.16	0.16	0.30	0.5960		0.20	0.20	1.65	0.2314
X_1^2	1	7.10	7.10	13.20	0.0055*		3.11	3.11	25.73	0.0007*
X_2^2	1	0.021	0.021	0.039	0.8469		0.018	0.018	0.15	0.7113
X_3^2	1	1.86	1.86	3.46	0.0959*		2.78	2.78	23.01	0.0010*

*Values of 'Prob > F' less than 0.05 indicate model terms; NA implies that the co-efficient are not applicable for linear model

Fig. 4 Effect of PO_4^{3-} and DIWW variables on $\text{COD}_{(\text{Removal } \%)}$ by *C. pyrenoidosa* in 3D plot **Fig. 5** Effect of NO_3^- and DIWW variables on total phosphorus (TP) removal by *C. Pyrenoidosa* in 3D plot

Nitrogen removal efficiency data suggest that linear (X_1 & X_2), interactive (X_1X_2) and quadratic (X_1^2) model terms were significant (F value of 11.47) as given in Fig. 6. When the added P was greater than or equal to 4 mg L⁻¹, nitrogen removal efficiency reached > 99% which authenticates the findings of other studies. The efficiency of nitrogen removal decreased the limitation of phosphorus which influences the algal growth. TN and TP could be analyzed and optimized by Eq. (8). For TN during the growth of *C. pyrenoidosa* uptake nutrient (N/P) was not necessarily the same as the constituent of algae.

$$Y_{(\text{TN } \%)} = 95.93 - 4.47X_1 - 5.62X_2 - 1.38X_3 - 4.58X_1X_2 + 2.21X_1X_3 + 0.28X_2X_3 - 5.83X_1^2 + 1.61X_2^2 - 2.11X_3^2 \quad (8)$$

Fig. 6 Effect of NO_3^- and DIWW variables on total nitrogen (TN) removal by *C. pyrenoidosa* in 3D plot

Effect of optimized process variables on algal productivity

Biomass productivity

The combined influence of DIWW, NO_3^- and PO_4^{3-} was found synergistic for biomass productivity of *C. pyrenoidosa*. The interrelationship of biomass productivity and variable concentration of DIWW, NO_3^- and PO_4^{3-} is well presented by the 3D plot (Fig. 7). The prob > F values for the linear (X_1 & X_2), quadric (X_1^2 , X_2^2 , and X_3^2) and interactive (X_2X_3) effects of DIWW, NO_3^- and PO_4^{3-} on growth of *C. pyrenoidosa* were found significant. This observation also suggests that DIWW concentration (trace X_1) had the least

Fig. 7 Effect of NO_3^- and DIWW variables on Biomass productivity of *C. pyrenoidosa* in 3D plot

effect on algal biomass weight. Alternatively, *C. pyrenoidosa* dry weight was greatly influenced by NO_3^- (increasing throughout the range; trace X_2) and quite sensitive to PO_4^{3-} (trace X_3). It should be noted though that the optimal range of nutrients (NO_3^- and PO_4^{3-}) lies outside the range of experiment values. The lack of fit test with p value of 0.05 showed that the model is satisfactorily fitted with the experimental design data (Abinandan and Shanthakumar 2016).

When R^2_{pred} values were compared to the actual values of biomass yield for *C. pyrenoidosa* R^2_{adj} was 0.948, indicating 94.8% model variability. The predicted and experimental values suggest that data fitted well with the RSM model and gives an accurately estimated response for the system using Eq. (9). Predicted and actual responses of lipid content are given in Fig. 3d. On the other hand, the residual response versus predicted responses showed a structure less plot suggesting that the model is acceptable and does not show any violation.

$$Y_{\text{Biomass}} (\text{mg/L}) = 0.85 + 0.086X_1 + 0.22X_2 + 6.99 \times 10^{-4}X_3 + 0.05X_1X_2 + 0.052X_2X_3 + 0.19X_1^2 + 0.047X_2^2 + 0.1X_3^2. \quad (9)$$

Lipid content

Lipid productivity of *C. pyrenoidosa* was greatly influenced by different DIWW combinations with nitrate and phosphate, which is shown in Fig. 8. The observation revealed higher lipid accumulation was found with lower concentration of these variables. It was found that higher levels of nitrate and phosphate had an inhibitory effect independently on lipid accumulation. However, the increased and positive value of the interactive effect

Fig. 8 Effect of NO_3^- and PO_4^{3-} variables on Lipid content removal by *C. Pyrenoidosa* in 3D plot

coefficient for DIWW, NO_3^- and PO_4^{-3} suggests that the accumulation of lipids was enhanced with increasing levels of selected variables. These results indicate that DIWW, NO_3^- and PO_4^{-3} ratio have a significant effect on lipid content in *C. pyrenoidosa*. The predicted values of lipid content calculated using the quadric Eq. (10) ranged between 11.16–56.62% (dry basis). The experimental design model observed that the effects of the linear terms (X_1 & X_2), quadratic terms (X_1^2 and X_2^2) and interactive terms (X_2X_3) were highly significant ($p < 0.05$). However, linear term (X_3), quadric term (X_2^2) and interactive term (X_1X_2 & X_1X_3) did not affect lipid content significantly ($p > 0.05$). The combined influence of DIWW, NO_3^- , and PO_4^{-3} can be represented in terms of second order polynomial Eq. (10), which is represented as:

$$Y_{\text{lipid}} (\%) = 28.5 - 1.02X_1 - 2.61X_2 - 0.3X_3 + 0.5X_1X_2 - 0.25X_1X_3 - 0.88X_2X_3 - 1.47X_1^2 + 0.42X_2^2 - 0.7X_3^2 \quad (10)$$

The significant $\text{prob} > F$ values for the linear, quadric and interactive effects of DIWW, NO_3^- and PO_4^{-3} , were evaluated. Moreover, the F value of 23.28 demonstrated that the model was meaningful, as revealed by a p value < 0.0001 , which supported the data for model validation. From the analysis of data, the R^2_{pred} of 0.61 was in good agreement with the R^2_{adj} of 0.92 in Fig. 3e and the data was well explained by this model. It is well known that the deprivation of NO_3^- increases lipid content in microalgae by two-fold (Simionato et al. 2013). Some researchers observed that maximum lipid content and biomass production occur at different values of the same parametric response. Hence, it is unlikely that maximum values for both responses can be found within the same experimental set-up. The microalgal biochemical system is similar to that of photosynthetic plants, in that it depends on macro- and micro-nutrient supply (Karemore et al. 2013). Advantages of coupling algal growth with wastewater treatment are that the wastewater provides nutrients for algal growth while at the same time saves freshwater and promotes lipid accumulation for bio-fuel production. Whilst some of the nutrients found in wastewater are growth limiting, others are involved in several enzymatic reactions for metabolite biosynthesis (Karemore et al. 2013).

FAME profile of transesterified bio-oil

Fatty acid composition of lipid from the optimization experiments with the highest lipid productivity in 15th run revealed that lipid profile of *C. pyrenoidosa* is composed of saturated, monounsaturated and polyunsaturated fatty acids. Fatty acid

Table 6 FAME composition in biodiesel from *Chlorella pyrenoidosa*

FAME	Carbon to double bond ratio	Concentration (%)
Methyl decanoate	C11:0	4.51
Methyl myristate	C14:0	1.28
Myristoleic acid methyl ester	C15:0	1.34
Methyl pentadecanoate	C16:0	3.13
Methyl palmitate	C17:0	0.80
Methyl arachidinate	C20:0	0.40
Total saturated		11.46
Cis-10 pentadecanoic	C15:1	1.08
Methyl palmitoleate	C16:1	6.02
Cis-9-oleic acid methyl ester	C18:1	3.71
Methyl linolenate	C18:2	2.50
Alpha linolenic acid	C18:3	0.86
Methyl cis-eicosatetraenoic acid	C21:4	0.94
Methyl erucate	C22:1	0.29
Docosahecanoic acid	C22:6	35.54
Methyl nervonate	C24:1	37.60
Total unsaturated		88.54
Total FAME		100%

profile influences the quality of biodiesel such as oxidative stability, cetane number, kinematic viscosity, iodine value, acid value etc. (Islam et al. 2013). Table 6 revealed that the percentage of unsaturated FAME is (88.54%) remarkable in comparison to that of saturated FAME (11.46%). The amount of fraction of saturated FAME is found between 0.40 to 4.51% while the amount of fraction of unsaturated FAME is between 0.29 and 37.60%. Table 6, summarized that the chain length of fatty acids comprising biodiesel in *C. pyrenoidosa* was between C11–C24. In case of saturated FAME, the major fatty acids were methyl decanoate and methyl pentadecanoate, while other saturated fatty acids were found below 2% in concentration. The dominant unsaturated fatty acids were methyl nervonate, docosahecanoic acid, methyl linolenate, oleic acid methyl ester, methyl palmitoleate, while other unsaturated fatty acids were found below 2%. The ideal mixture of fatty acids has been suggested to be C16:1, C18:1 and C14:1 in the ratio of 5:4:1. It has been found that such biodiesel composition would have the properties of very low oxidative potential (Schenk et al. 2008; Kondo et al. 2016). In present study, ratio of C16:1, C18:1 and C14:1 was found to be ~5:3:1. Thus C18:1 concentration of *C. pyrenoidosa* has to be improved by manipulating the culture condition. In this study, the biodiesel is found to have higher unsaturated fatty acid concentration than that of saturated fatty acids, which represents the reduced oxidative stability of biodiesel. Generally, oxidative stability is

influenced by extent of unsaturation and higher unsaturation causes the poor oxidative stability (Hoekman et al. 2012).

Earlier investigation performed by Schenk et al. (2008) strengthens the findings of present study and reported presence of fatty acids from C11 to C24. They found C14:0, C16:0 and C17:0 as a major saturated fatty acid in *C. pyrenoidosa*. In a recent study conducted by Shen et al. (2016) also reported higher concentration (2–3 times) of unsaturated fatty acids than that of saturated fatty acids in biodiesel obtained from *C. pyrenoidosa* in different culture conditions. Presence of palmitic acid, oleic acid, and alpha linolenic acid also evaluated and reported for qualitative assessment of biodiesel at commercial level because, Palmitic acid positively correlated with CN and negativity correlated with oleic acid and alpha linolenic (Gopinath et al. 2010). Cetane number (cetane rating) is an indicator of the combustion speed of diesel fuel and compression needed for ignition and described the low oxidative stability, which increase the rancidity of biodiesel.

Conclusion

The highest lipid content (34.41%) and highest biomass productivity (1.54 g L^{-1}) in *Chlorella pyrenoidosa* was obtained under the moderate concentration of dairy industry wastewater (50%). Results clearly revealed the significance of nitrogen stress that increased the lipid content approximate three times compared to normal BG 11 nutrient medium. Analysis of experimental data using response surface methodology revealed that nitrogen is the most influential factor followed by dairy industry wastewater concentration for lipid(bio-oil) as well as biomass productivity. The $\text{Prob} > F$ value for X_3 was found higher than 0.05 for lipid and biomass productivity, which indicates that phosphorus have non-significant influence on the observed responses. *C. pyrenoidosa* was found to uptake > 99% of total phosphorus and nitrogen from the manipulated medium while maximum efficiency to reduce COD was 43.5%. Consequently, *C. pyrenoidosa* can be recommended for nutrient uptake during the secondary wastewater treatment process. FAME analysis of simultaneously transesterified algal oil, supports its quality for commercial applicability with higher level unsaturated fatty acid concentration.

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Compliance with ethical standards

Conflict of interest The authors of this manuscript declare that there is no conflict of interest.

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