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Multi-objective optimization of media components for improved algae biomass, fatty acid and starch biosynthesis from *Scenedesmus* sp. ASK22 using desirability function approach

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ABSTRACT

In this study, we sought to improve medium composition for enhanced biomass, lipid and starch content by using response surface methodology and composite desirability function approach for the heterotrophic cultivation of *Scenedesmus* sp. ASK22. Maximum biomass yield observed was 5.02 ± 0.1 g L^{-1} and 4.67 ± 0.12 g L^{-1} for individual and multi-response respectively. Lipid and starch accumulation for individual response and multi-response were found to be 30.72 ± 0.23 (% wt.) and 37.10 ± 2.71 (% wt.) and 29.46 ± 0.17 (% wt.) and 31.06 ± 0.17 (% wt.) respectively. The Lipid and starch productivity in BG11 medium increased to 13.38 and 28.59-fold for individual optimized conditions and 18.20 and 31.97-fold for multi-response optimized conditions respectively. These results propose *Scenedesmus* sp. ASK22 strain to be a potential candidate for bio-fuel production.

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1. Introduction

Over a few decades, scientific communities from all over the world have recognized microalgae as a nontoxic, renewable, biodegradable source of biofuel feedstock and other industrial commodities [1]. As compared to the first and second-generation biofuel feedstock (corn, sugar beet, palm, canola, sweet sorghum and straw, wood), microalgae-based biofuel has been perceived to be a superior replacement and offers great advantages over currently available feedstock [2]. Microalgae can be cultivated easily, can remediate wastewater, grow and reproduce themselves using light energy and store food in the form of organic compounds viz. starch, proteins and lipid [3–5]. Microalgae complete their entire lifecycle within a few days [6]. Besides this, microalgae growth rate and chemical composition can easily be manipulated by changing the nutritional composition or environmental conditions as per process requirements [7]. Furthermore, the microalgae show a broad range of species living in diverse environmental conditions, making it possible to select appropriate algal species to the local environment, which is infeasible to do with other available biofuel feedstocks [2]. Despite immense potential and concerted research efforts, an economic feasibility issue is faced in industrial microalgae cultivation because of the expensive mineral salt medium and the production of low levels of biomass under the photoautotrophic mode of cultivation [8,9].

To solve these issues number of novel methods have been applied such as screening of oleaginous microalgae, lipid and biomass accumulation coupled with wastewater remediation, two-stage cultivation, co-cultivation of microalgae with yeast and bacteria, supplementation of phytohormones and others chemical additives (Azide, Brefeldin-A and surfactant). Further to this, optimization of process parameters including media composition, cultivation conditions, and the types of metabolism also play a pivotal role in making the process economically more viable [10]. Therefore, it is important to utilize specific as well as appropriate conditions for cultivation to attain higher lipid/starch production efficiency for the microalgae species.

The heterotrophic metabolism offers several advantages over

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the autotrophic metabolism, for example, it may reduce the culture batch length to achieve desired cell density, does not require light and it has low doubling time, high biomass productivity and reduced cost of downstream processing [11–13]. Therefore, heterotrophic cultivation could be considered as a potential cultivation method for viable algal biomass production to obtain a variety of bioproducts [11].

One of the significant factors that influence the growth and metabolic composition of microalgae is the composition of nutrient media [14]. The heterotrophic cultivation of microalgae is generally governed by types and source of carbon, nitrogen and phosphorus added to the medium. Therefore, the optimization of medium constituents is of great significance to achieve a high cell density [11,15]. It is notable that the medium optimization is done by two different ways viz. traditional "one factor at a time/empirical" and statistical methods. The traditional empirical method is sluggish and ineffective to contemplate the collective effect of all the entities involved [16]. The culture media optimization could be refined by using the statistical methodology, which includes factorial design and regression analysis. This helps in the assessment of variables by building models to determine interaction and select the optimal condition of variables for a desirable response [11,17]. This procedure has been broadly used for optimization of several process parameters to enhance individual response viz. algal biomass, lipid, starch and other value-added products. To the best of our knowledge, no studies have been done using multi-response optimization to perk up the lipid and starch productivity concurrently in *Scenedesmus* sp..

Therefore, the main intent of this preliminary study was to screen the most suitable mineral salt medium for *Scenedesmus* sp. ASK22, followed by the screening of favourite carbon and nitrogen source for the heterotrophic cultivation of *Scenedesmus* sp. ASK22. Further to this, optimization of selected media component (carbon and nitrogen) including dipotassium hydrogen phosphate (K_2HPO_4) was utilized to enhance growth, lipid and starch productivity individually and in a cumulative manner of *Scenedesmus* sp. ASK22 by using Response Surface Methodology (RSM).

2. Material and methods

2.1. Microalgae strain and cultivation

A unicellular green microalgal species, *Scenedesmus* sp. ASK22 (Accession No. MF945631) was used in this study [18]. The algal culture preservation and inoculum preparation for all experiments were performed in modified-BG11 medium (pH 7.4 ± 0.1) [19] by incubating at 30 °C with a 12:12 light and dark cycle (light intensity of 40 μmol m^{-2} s^{-1}). Prior to inoculation, the mineral salt medium was autoclaved at 121 °C for 15 min. The cultivation was performed in 250 ml flask with 100 mL working volume. Inoculation was performed at the concentration of ≈60–70 mg L^{-1}. Intermittent shaking was frequently performed to avoid the adherence of algae on the bottom of the flask. The samples were taken at regular intervals for the estimation of biochemical parameters.

2.2. Analytical methods

2.2.1. Estimation of dry cell weight, lipid productivity and carbohydrate productivity

The dry cell weight was determined gravimetrically by harvesting microalgae at the end of 7 days batch cultivation. Lipid content, biomass-, lipid-, starch productivity and specific growth rate were calculated using Eqs. (1)–(5), respectively.

$$\text{Lipid content (LC) (\% wt.)} = (W \times 100)/B \quad (1)$$

$$\text{Biomass productivity (BP) (mg } L^{-1} d^{-1}) = X_t - X_0 / t \quad (2)$$

$$\text{Lipid productivity (LP) (mg } L^{-1} d^{-1}) = \text{LC (\% wt.) x BP x 0.01} \quad (3)$$

$$\text{Starch productivity (SP) (mg } L^{-1} d^{-1}) = \text{SC (\% wt.) x BP x 0.01} \quad (4)$$

$$\text{Specific growth rate } (\mu\ (d^{-1})) = \frac{\ln A_2 - \ln A_1}{t_2 - t_1} \quad (5)$$

where, Xt is the final concentration of cells at the end of batch (mg L^{-1}). Xo is the initial biomass concentration (mg L^{-1}) and, t is the total duration of batch cultivation (day). LC and CH are the lipid and carbohydrate content (% wt.), respectively. *W* and *B* are the weight of dried lipid (mg) and biomass (mg), respectively. A_1 and A_2 are the absorbance at 680 nm at the time t_1 and t_2, respectively.

2.2.2. Lipid extraction and fatty acid composition analysis

Total lipids were extracted from the dried algal cell biomass as per the Bligh and Dyer method [20]. The quantitative estimation of total lipid content (% wt.) was performed gravimetrically [17]. Briefly, a known amount (50 mg) of dried algal biomass was extracted in methanol: chloroform (2:1, v/v); suspension was homogenised using vortex for 5 min followed by centrifugation at 5000*g* for 15 min. The extraction was repeated four times until the biomass became colourless. Fatty acid derivatization of microalgal oil was carried out employing a 5:1 M ratio of methanol: oil in the presence of conc. H_2SO_4, at 70 °C for 3 h. Fatty acid methyl esters composition was analysed by GC-MS using DB-5MS (5% phenyl)-methylpolysiloxane non-polar column (30 m × 0.25 mm x 0.25 μm) [17]. Fatty acid composition analysis of microalgae oil was carried out by using the protocol established by Breuer et al. [21].

2.2.3. Starch extraction and quantification

The starch was extracted and estimated, using the procedure given by MaCready et al. [22]. A known amount (50 mg) of dried algal biomass and 20 ml of 80% (v/v) ethanol were mixed together and incubated for 12 h at room temperature. The mixture was centrifuged at 2000 RPM for 15 min. The collected residual biomass was mixed with 5 ml dH_2O and subsequently, 6.5 ml of 52% perchloric acid solution and the content were mixed vigorously for 10 min. The entire content was centrifuged for 20 min at 2000 RPM. The supernatant was collected and decanted and the entire process was performed thrice. The supernatant of each step was collected and the total volume was made up to 50 ml by adding distilled water. The mixture was then allowed to filter out through filter paper (Whatman No. 42). An aliquot (1.0 ml) of this filtrate was analysed as per the phenol-sulfuric acid method for calculating the starch content [23]. The quantity of starch was estimated in terms of glucose equivalent and to convert the values of glucose to starch the factor of 0.9 was taken into consideration. The quantity of starch was expressed in terms of % dry wt. of microalgae.

2.3. Design of experiments

The experimental design employed initial screening of best growth medium out of five available media (BG11, M_4N [24], BBM [25], Fog [26] and SK [27] (compositions of media shown in Table S1). In the next step, best carbon sources (glucose, sucrose, lactose, glycerol, maltose and sodium acetate), nitrogen sources (yeast extract, meat extract, urea, sodium nitrate and ammonium nitrate) were screened. Based on preliminary screening, the three independent variables viz. glucose ($C_6H_{12}O_6$), urea and dipotassium

Table 1
The levels of variables for experimental design.

Variables	Levels				
	-α	−1	0	+1	+α
A: Nitrogen: Urea (g L^{-1})	0.74	1.25	2.00	2.75	3.26
B: Phosphorus: K_2HPO_4 (mg L^{-1})	6.36	20.00	40.00	60.00	73.64
C: Carbon: Glucose (g L^{-1})	6.59	10.00	15.00	20.00	23.41

hydrogen phosphate (a component of BG11 media) were chosen for further optimization using central composite design (CCD). The concentration of variables urea (A), K_2HPO_4 (B) and glucose (C) was carefully chosen as independent experimental variables and their levels were coded as −1 (low), 0 (central point) and +1 (high) by using Eq. (6).

$$X_i = \frac{X_i - X_{o,i}}{\Delta X_i}, \; i = 1, 2, 3.....k \tag{6}$$

where, x_i and X_i are the coded and encoded values of each variable, $X_{o,i}$ indicates the encoded value of each variable at the centre point and, ΔX_i, is the step change value.

The dry cell weight, lipid- and starch content were considered as dependent variables. The range and levels of independent variables are shown in Table 1. The experimental design was a 2^3 full factorial consisting of 20 trials, containing six axial points (±α from the centre), eight factorial points (±1 from the centre point) and six replicates (at the centre). Experiments at the central points were run to determine the curvature and to compensate the lack of fit values which indicate the significance of the model (Table 2) [28,29].

The value of α was calculated by using Eq. (7).

$$\alpha = 2^{k/4} \tag{7}$$

where, k is the number of factors; therefore, the value of α is equal to $(2)^{3/4} = 1.68$.

The kinetics was explained by the second order polynomial model as described by Eq. (8).

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i x_i + \sum_{i=1}^{2} \sum_{j=i+1}^{3} \beta_{ij} x_i x_j + \sum_{i=1}^{3} \beta_{ii} x_i^2 + \varepsilon \tag{8}$$

where Y is the response, β_0 β_i β_{ii} and β_{ij} are the regression coefficients for intercept, linear effects, and double interactions respectively. The x_i and x_j represent the coded independent variables. The analysis of experiments was performed using the Design of experiments software (Design Expert®, trial version 11.0, Stat-Ease, Inc., Minneapolis, USA), based on the RSM. In Design Expert, the matrix operation was the main feature to find the coefficients of the regression equation. Analysis of variance (ANOVA) was conducted on the actual level of variables to identify the effects of individual variables.

2.4. Multi-response optimization

For this study, there were three responses (dry cell weight, lipid- and starch content), therefore the multi-response optimization by desirability function approach was used to optimize *Scenedesmus* sp. ASK22 growth. Simultaneous optimization combines all the three-response requirements into one cumulative requirement. Individual desirability d_i was calculated by using Eq. (9).

$$d_i = \begin{cases} 0 & if\ Y^i \le Y^{i-min} \\ \left(\dfrac{Y^i - Y^{i-min}}{Y^{i-max} - Y^{i-min}}\right)^r & if\ Y^{i-min} < Y^i < Y^{i-max} \\ 1 & if\ Y^i \ge Y^{i-max} \end{cases} \tag{9}$$

where, Y^i is response value; $Y^{i\text{-}min}$ and $Y^{i\text{-}max}$ is minimum and maximum acceptable value of response respectively; i and r is a weight and positive constant used to determine the scale of desirability. In the multi-response optimization, desirability function transforms each response to a corresponding desirability value between 0 and 1. All the individual desirability functions are combined to form a composite desirability function (D) which converts a multi-response into a single response. The individual desirability functions are combined to obtain the composite desirability, as defined by Eq. (10).

$$D = (d_i x\ d_2\ x\ d_3.....)^{1/k} \tag{10}$$

where, $0 \le D \le 1$ and k is the number of responses. If all the quality characteristics reach their ideal values, the desirability d_i is 1.

The composite desirability lies between 0 and 1 representing the closeness of a response to its ideal value. If the response falls within the ideal intervals or the response reaches its ideal value, the desirability is 1. Moreover, when the response falls within the tolerance interval but not the ideal interval, the desirability lies between 0 and 1 [30].

2.5. Experimental verification of models and statistical analysis

To verify the developed models, confirmatory experiments were performed at optimum levels of variables for maximization of biomass, lipid- and starch content individually to validate the developed models. The experiments were performed independently in duplicates. The results are expressed as the mean value of two independent replicates with error bars representing the standard deviation for each set of experiment. Design Expert®, trial version 11.0, USA software was used to obtain the analysis of variance and regression analysis from experimental data.

3. Results and discussion

3.1. Preliminary screening of medium, carbon and nitrogen source

The *Scenedesmus* sp. ASK22 is an indigenous unicellular oval microalga which was isolated by the authors from the aeration tank of the dairy effluent treatment plant [18]. The high specific growth rate is a direct measure of high cell yield and metabolites within a limited time. The *Scenedesmus* sp. ASK22 growth in BG11 ($\mu = 0.315$ d^{-1}) was highest among all five selected media followed by Fog ($\mu = 0.278$ d^{-1}) > SK ($\mu = 0.272$ d^{-1}) > BBM ($\mu = 0.168$ d^{-1}) > M_4N ($\mu = 0.116$ d^{-1}) (Fig. S1(a)). It revealed the influence of different media compositions on the growth of *Scenedesmus* sp. ASK22. Similarly, Kirrolia et al. [31] also tested different mineral salt medium for *Chlorella* sp. growth and of all the tested mineral salt media, BG-11 was the favourite medium for its biomass production. Dayananda et al. [32] observed similar results for green microalga *Botryococcus braunii* in the BG-11 medium among all tested growth medium. Wong et al. [33] investigated various growth medium on *Chlorella vulgaris* and concluded Bold basal medium (BBM) to be the best for specific growth rate (0.279 ± 0.001 d^{-1}) and biomass productivity (114.208 ± 0.850 mg L^{-1} day^{-1}).

Few algal species can consume organic carbon sources and

Table 2
Central composite design matrix with observed and predicted values.

Run	Encoded Value			Dry cell weight (g L^{-1})		Lipid content (% wt.)		Starch (% wt.)	
	A: Nitrogen (g L^{-1})	B: Phosphorus (mg L^{-1})	C: Carbon (g L^{-1})	Observed value	Predicted value	Observed value	Predicted value	Observed value	Predicted Value
1[a]	2	40	15	4.65 ± 0.24	4.82	31.02 ± 0.02	30.57	32.50 ± 0.08	32.22
2[a]	2	40	15	4.98 ± 0.12	4.82	30.12 ± 0.00	30.57	32.45 ± 0.03	32.22
3	1.25	20	20	3.75 ± 0.17	3.68	29.53 ± 0.03	29.15	31.02 ± 1.37	31.52
4	2.75	20	10	2.96 ± 0.08	2.94	27.60 ± 0.18	27.45	26.16 ± 1.05	25.54
5	1.25	20	10	3.61 ± 0.19	3.71	25.92 ± 1.02	25.42	15.96 ± 1.86	16.56
6	1.25	60	10	3.38 ± 0.10	3.42	30.85 ± 0.56	30.69	28.67 ± 1.81	28.08
7[a]	2	40	15	4.63 ± 0.06	4.82	30.56 ± 0.71	30.57	32.67 ± 2.08	32.22
8[a]	2	40	15	4.96 ± 0.04	4.82	30.10 ± 1.12	30.57	32.63 ± 1.23	32.22
9	0.74	40	15	2.78 ± 0.11	2.72	29.72 ± 0.74	30.30	26.40 ± 1.39	25.73
10[a]	2	40	15	4.93 ± 0.03	4.82	31.07 ± 1.18	30.57	31.78 ± 0.36	32.22
11	3.26	40	15	3.22 ± 0.12	3.22	26.58 ± 0.32	26.69	22.18 ± 1.01	22.90
12	2	40	6.59	3.51 ± 0.15	3.39	28.56 ± 0.21	28.87	17.53 ± 1.43	18.19
13	2	73.64	15	4.13 ± 0.02	4.06	25.80 ± 0.04	25.92	37.24 ± 2.03	37.92
14	2.75	60	10	3.31 ± 0.16	3.42	27.09 ± 0.17	26.98	24.98 ± 2.34	24.45
15	2.75	20	20	4.29 ± 0.07	4.28	28.91 ± 0.32	28.58	31.24 ± 1.27	31.79
16[a]	2	40	15	4.78 ± 0.10	4.82	30.65 ± .011	30.57	31.27 ± 2.46	32.22
17	2	40	23.41	3.95 ± 0.21	4.02	28.36 ± 1.20	28.75	33.68 ± 0.27	33.06
18	2	6.36	15	4.36 ± 0.03	4.38	25.51 ± 1.36	26.09	32.74 ± 0.38	32.11
19	2.75	60	20	4.25 ± 0.05	4.19	23.10 ± 0.82	23.11	27.81 ± 0.87	27.18
20	1.25	60	20	2.77 ± 0.24	2.83	29.76 ± 0.03	29.42	38.93 ± 0.65	39.52

[a] Experiments performed at the central value. The observed values of responses are represented as mean value ± standard deviation.

conduct heterotrophic metabolism [34]. Once the suitable medium was identified, we tested for the best organic carbon source. The heterotrophic cultivation of *Scenedesmus* sp. ASK22 was tested for different organic carbon sources (viz. sodium acetate, glycerol, maltose, lactose, sucrose and glucose) but highest specific growth rate ($\mu = 0.451$ day^{-1}) was achieved with BG-11 supplemented with glucose (10 g L^{-1}) (Fig. S1(b)). Ren et al. [35] reported that *Scenedesmus* sp. R-16 with optimum carbon source (glucose; 10 g L^{-1}), yielded higher biomass (3.43 g L^{-1}). Dittamart et al. [36] also found similar results for *Scenedesmus* sp. AARLG022 with biomass yield of 2.78 ± 0.86 g L^{-1} in the medium supplemented with 0.5M glucose. Similarly, glucose remarkably influenced the growth and lipid yield of *Scenedesmus quadricauda* and obtained 3.39 g L^{-1} biomass (under 5 g L^{-1} glucose) on day 7 with high specific growth rate (0.572 ± 0.024 d^{-1}) [37].

Nitrogen is the fundamental constituent of the protein and nucleic acids and is essential for microalgal cell division and growth. Inorganic nitrogen in microalgae is rapidly assimilated and recycled within the cell to meet changing physiological needs [7]. Microalgae growing in nitrogen-depleted medium tend to divert their fixed carbon pool to accumulate starch/carbohydrate/lipids. This in turn results in increase lipid/protein or starch/protein ratio [38]. The screening for best nitrogen source suggested urea (0.636 d^{-1}) to be the most suitable nitrogen source, among all tested nitrogen sources followed by sodium nitrate ($\mu = 0.551$ d^{-1}) >potassium nitrate ($\mu = 0.440$ d^{-1}) > ammonium acetate ($\mu = 0.396$ d^{-1}) > ammonium chloride ($\mu = 0.325$ d^{-1}) (Fig. S1(c)). Glass et al. [39] suggested that urea as dissolved organic nitrogen source, can be effectively assimilated by algal cells through the glutamine synthetase-glutamate synthase pathway. In a similar investigation, Crofcheck et al. [40] tested the effect of nutrient composition on the growth rate of *Chlorella vulgaris* and *Scenedesmus acutus*. Urea-based medium worked better for *Scenedesmus acutus* whereas, KNO_3 based medium was found suitable for *Chlorella vulgaris*. Phosphorus is the constituent element of ATP and is of great importance for phosphorylation, which has significant relevance to the cell growth and metabolism of microalgae. Phosphate starvation in the media results in suppression of ATP synthesis and assimilation of carbon dioxide [41]. However, an increase in phosphate in the medium, channelizes the pathway for synthesis of more ribulose-5-phosphate. This may be a reason behind increasing biomass yield by increasing phosphate concentration. Previous studies [42] investigated and concluded that phosphorus plays a significant role in lipid production under nitrogen starved condition in *Chlorella vulgaris*. Lipid accumulation was 58.39 mg $L^{-1}d^{-1}$ after 14 days batch cultivation under highest initial phosphorus supply (35 mg $L^{-1}d^{-1}$). *Chlorella pyrenoidosa* cultivated in phosphorus deficient medium showed it to be critical in biomass production (0.55 g L^{-1}) in comparison to the control (0.88 g L^{-1}). Similarly, Tourang et al. [41] observed a severe decrease in *Spirulina platensis* growth under decreasing phosphate concentration. However, Markou et al. [43] observed no considerable effect on biomass productivity of *Arthospira platensis*.

3.2. Optimization of medium nutrient components to maximize the biomass production, lipid- and starch content using CCD

Further optimization of heterotrophic cultivation of *Scenedesmus* sp. ASK22 was done for glucose, urea and K_2HPO_4 (phosphorus source and component of BG11) using modified-BG11 as a basal medium. The application of CCD-RSM approach yielded the following second order polynomial equations (expressed in terms of coded value). Dry cell weight (Y_1 (Eq. (11))), lipid content (Y_2 (Eq. (12))) and starch content (Y_3 (Eq. (13))) as the functions of three independent variables-nitrogen (urea (A)), phosphorus (K_2HPO_4 (B)) and carbon (glucose (C)) is as follows:

$$\text{Dry cell weight}\left(Y1\left(\text{g L}^{-1}\right)\right) = 4.82 + 0.1494A - 0.0942B + 0.186C + 0.19AB + 0.3425AC - 0.1425BC - 06541A^2 - 0.214B^2 - 0.396C^2 \quad (11)$$

$$\text{Lipid content}(Y2(\%\ \text{wt.})) = 30.57 - 1.07A - 0.0492B - 0.0363C - 1.43AB - 0.65AC - 1.25BC - 0.7316A^2 - 1.61B^2 - 0.622C^2 \quad (12)$$

$$\text{Starch content}(Y3(\%\ \text{wt.})) = 32.22 - 0.8411A + 1.73B + 4.42C - 3.15AB - 2.18AC - 0.8813BC - 2.79A^2 + 0.9888B^2 - 2.33C^2 \quad (13)$$

Experimental setup included CCD with five coded levels for all three variables. Their respective range is given in Table 1. The complete CCD matrix with a range of variables and the observed and predicted values of responses viz. dry cell weight (gL^{-1}), lipid content (% wt.) and starch content (% wt.) are presented in Table 2. The experimental data obtained for *Scenedesmus* sp. ASK22 biomass concentration, lipid- and starch content were dependent on the combination of urea, K_2HPO_4 and glucose concentrations. The optimization of the medium to achieve optimal final biomass concentration, lipid- and starch content was conducted individually.

The statistical testing of the quadratic regression equation models was performed using the analysis of variance (ANOVA), which is required to access the significance and adequacy of the model. The results summary of ANOVA for the selected responses is shown in Table 3. Here, the ANOVA of the regression model is highly significant as indicated by the high F value (dry cell weight (61.10), lipid content (41.23) and starch content (103.77)) with $p = 0.01$ for all three responses individually. Furthermore, the lack of fit F-value for dry cell weight (0.7638), for lipid- (0.2595) and for starch content (0.1065) implies that it is not significant.

The goodness of fit of the regression equation is tested by examining the adjusted determination coefficient (R^2_{Adj}). The results showed high R^2_{Adj} (0.9661, 0.9501 and 0.9799 for equation (10)–(12), respectively) indicating a reasonable agreement between observed and predicted values for all three responses and suggesting that the proposed model equation provides satisfactory and accurate results. In addition, the high value of multiple correlation coefficients and Adeq. precision for biomass concentration (0.9821 and 21.76), lipid- (0.9738 and 21.44) and starch content (0.9894 and 39.04) also indicates the suitability of models in terms of independent variables (Table 3). The results showed that A, B, C, AB, AC, BC, A^2, B^2 and C^2 are significant model term for dry cell weight (Y_1) and starch content (Y_3), respectively. Whereas, A, AB, AC, BC, A^2, B^2 and C^2 are significant model term for lipid content (Y_2). The plot of the studentized residuals versus the run number of the dry cell weight, lipid content and starch content show that the residuals for both dependent variables scattered randomly around ±4.1479, which indicated that the observed data fits well with these models (Fig. 1 (b, d and f)).

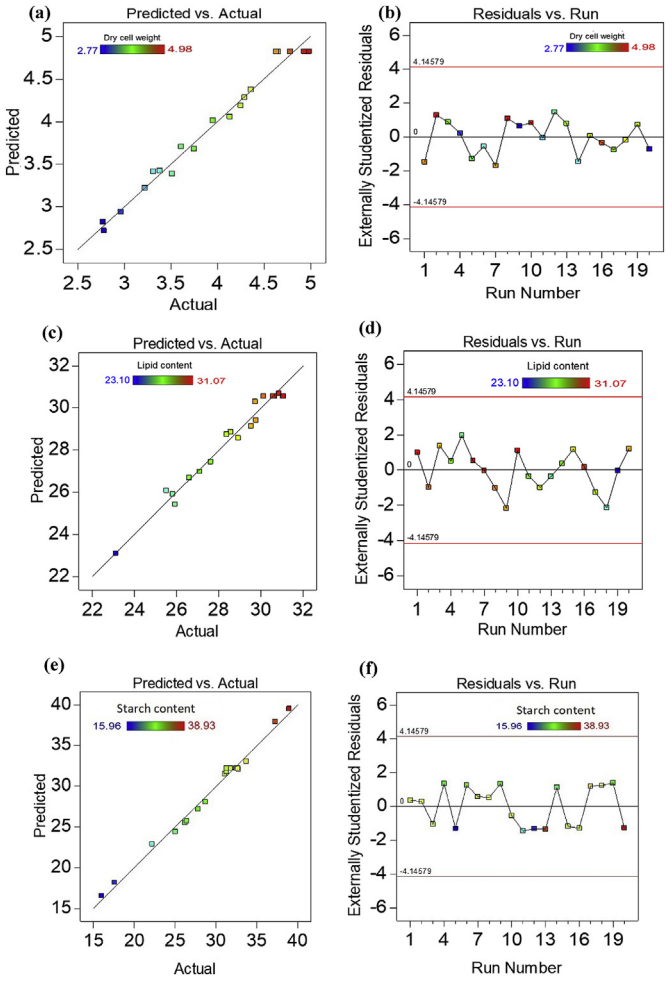

Fig. 1. The plot of predicted value versus the actual response for: dry cell weight (gL^{-1}) model (a), lipid content (% wt.) model (c), starch content (% wt.) model (e); internally studentized residuals versus run number for: dry cell weight (gL^{-1}) model (b), lipid content (% wt.) model (d), starch content (% wt.) model (ff).

The coefficient of variation (CV) is another statistical method which showed the reliability of these experiments. The lower CV indicates the higher reliability of the results. The results showed lower CV (3.45%, 1.75% and 2.83%) for dry cell weight, lipid- and

Table 3
ANOVA for response surface quadratic models of dry cell weight, lipid content and starch content.

Source	Dry cell weight (g L^{-1})[a]					Lipid content (% wt.)[b]					Starch content (% wt.)[c]				
	SS[d]	Df[d]	MS[d]	F-value	P value	SS[d]	Df[d]	MS[d]	F-value	P value	SS[d]	Df[d]	MS[d]	F-value	P value
Model	10.26	9	1.14	61.10	<0.0001	92.85	9	10.32	41.23	<0.0001	645.81	9	71.76	103.77	<0.0001
A	0.3047	1	0.3047	16.34	0.0024	15.70	1	15.70	62.72	<0.0001	9.66	1	9.66	13.97	0.0039
B	0.1212	1	0.1212	6.50	0.0289	0.0331	1	0.0331	0.1332	0.7237	40.71	1	40.71	58.87	<0.0001
C	0.4724	1	0.4724	25.32	0.0005	0.0180	1	0.0180	0.0721	0.7938	267.05	1	267.05	396.19	<0.0001
A.B	0.2888	1	0.2888	15.48	0.0028	16.47	1	16.47	65.83	<0.0001	79.57	1	79.57	115.07	<0.0001
A.C	0.9384	1	0.9384	50.31	<0.0001	3.38	1	3.38	13.51	0.0043	37.89	1	37.89	54.79	<0.0001
B.C	0.1625	1	0.1625	8.71	0.0145	12.50	1	12.50	49.95	<0.0001	6.21	1	6.21	8.98	0.0134
A^2	6.17	1	6.17	330.57	<0.0001	7.71	1	7.71	30.83	0.0002	112.52	1	112.52	162.72	<0.0001
B^2	0.6597	1	0.6597	35.37	0.0001	37.53	1	37.53	149.97	<0.0001	14.09	1	14.09	20.38	0.0011
C^2	2.26	1	2.26	121.17	<0.0001	5.58	1	5.58	22.28	0.0008	78.19	1	78.19	113.07	<0.0001
Residual	0.1865	10	0.0187			2.50	10	0.2503			6.92	10	0.6915		
Lack of fit	0.0627	5	0.0125	0.5058	0.7638	1.62	5	0.3244	1.84	0.2595	5.32	5	1.06	3.33	0.1065
Pure error	0.1239	5	0.0248			0.8807	5	0.1761			1.60	5	0.3196		

[a]C.V = 3.45%; R^2 = 0.9821; R^2 (predict) = 0.9350; R^2 (adjust) = 0.9661; PRESS = 0.6794.
[b]C.V = 1.75%; R^2 = 0.9738; R^2 (predict) = 0.8574; R^2 (adjust) = 0.9501; PRESS = 13.60.
[c]C.V = 2.83%; R^2 = 0.9894; R^2 (predict) = 0.9326; R^2 (adjust) = 0.9799; PRESS = 43.97.
[d] SS: Sum of square; Df: Degree of freedom; MS: Mean square.

starch content, respectively (Fig. 1 (a, c and e)). Therefore, it can be concluded that the mathematical models can be utilized for optimization of independent variables (nitrogen, phosphorus and carbon) and predicting dry cell weight, lipid- and starch content in case of heterotrophic cultivation of microalgae.

3.3. Relative effects of medium nutrient components on the biomass concentration, lipid content and starch content

The perturbation plot helps to compare the effect of all the factors on responses in the design space. The response is plotted by changing only one factor over its range while holding all the other factors constant. A steep slope or curvature in a factor shows that the response is sensitive to that factor. A relatively flat line shows insensitivity to change in that factor. Perturbation plot (Fig. S2- (a & c)) of all the three-factors clearly shows that nitrogen, phosphorus and carbon are the significant factors for *Scenedesmus* sp. ASK22 for dry cell weight and starch content. Carbon had a slight effect on lipid content as compared to nitrogen and phosphorus. On the other hand, nitrogen was the most significant factor for lipid content among all the three factors (Fig. S2(b)). On the other hand, starch accumulation was found to be highest at higher concentration of phosphorus and carbon and at low nitrogen concentration.

3.4. Evaluation of the mutual effect of medium nutrients on the biomass concentration, lipid- and starch content

The 2-D contour diagram was plotted to visualize the mutual interaction between medium components for all three responses (dry cell weight, lipid- and starch content). An elliptical nature of the contour plot shows significant interaction between variables, whereas, 2-D contour surface having circular plots indicate that the interaction between the variables under study is negligible. The centre eclipse in the contour diagram shows the maximum value of the predicted response in selected intervals of the variables [41]. Fig. 2(a–c) shows the 2-D contour diagram as a function of two variables, whereas the other variables have been kept at the centre point.

3.4.1. The mutual interaction of the variables for increased dry cell weight

All the tested interactions have a significant effect on *Scenedesmus* sp. ASK22 biomass production. The interaction between nitrogen and carbon was found to be very prominent followed by nitrogen and phosphorus and phosphorus and carbon (Table 3). The mutual effect of nitrogen with phosphorus and carbon on the biomass production of *Scenedesmus* sp. ASK22 is shown in Fig. 2 (a). The maximum dry cell weight (4.88 g L^{-1}) was obtained for nitrogen concentration between 1.88 g L^{-1} to 2.24 g L^{-1} and phosphorus concentration from 27.55 mg L^{-1} to 45.01 mg L^{-1} provided that the glucose concentration was >11.84 g L^{-1}. The interaction between nitrogen and carbon for biomass production indicates that the highest dry cell weight (4.87 g L^{-1}) could be obtained at the 2.14 g L^{-1} nitrogen and at carbon concentration ranging from 15.91 g L^{-1} to 16.68 g L^{-1}. The *Scenedesmus* sp. ASK22 biomass production was maximum (4.86 g L^{-1}) for the interaction between phosphorus and carbon, when glucose was 16.38 g $L^{-1}$and phosphorus was 34.36 g L^{-1} (Fig. 2a).

3.4.2. The mutual interaction of the variables for lipid content

The contour plot of the mutual interaction of nitrogen with phosphorous and carbon for the lipid content is shown in Fig. (2b). The results indicate that, maximum lipid content was obtained with nitrogen <1.25 g L^{-1}, phosphorus 41.45–55.57 mg L^{-1} and carbon maintained between 15.5 and 18.97 g L^{-1}. The interaction between phosphorus and carbon indicates highest lipid accumulation when phosphorus and carbon vary from 40 to 54.63 mg L^{-1} and 8.77–21 g L^{-1}, respectively. It is clear from Fig. (2b) that the interaction between nitrogen with phosphorus and phosphorus with carbon play a crucial role for *Scenedesmus* sp. ASK22 lipid content as compared to the interaction of nitrogen with carbon.

3.4.3. The mutual interaction of the variables for starch content

The contour plot showing the mutual interaction between nitrogen with phosphorus and carbon for *Scenedesmus* sp. ASK22 starch production indicates that the highest starch production (≈39 % wt.) was obtained at nitrogen 1.25–2.05 g L^{-1} and at a higher concentration of phosphorus (>58 mg L^{-1}) and carbon (>19.64 g L^{-1}) (Fig. 2c). The mutual interaction between all three variables is found to be significant in the case of starch production. Fig. 2 (c) shows that maximum starch content could be obtained at a higher concentration of phosphorus and carbon and at low concentration of nitrogen.

3.5. Individual and multi-response optimization of medium composition

A numerical method given by Mayers and Mongomery [29] was used to analyse the regression Eqs. (11)-(13). The optimum value of test variables (nitrogen, phosphorus and carbon) in coded units are as follows: Dry cell weight (A = −0.17, B = −0.26 and C = 0.33); lipid content (A = −0.95, B = 0.43 and C = −0.25) and starch content (A = −0.94, B = 0.94 and C = 0.85) with the corresponding $Y_1 = 4.88$ g L^{-1}, $Y_2 = 31.11$ (% wt.) and $Y_3 = 38.95$ (% wt.) respectively (Fig. 3). The actual values obtained by putting the respective value of A, B and C in equations (10)–(12) are: 2.13 g L^{-1} nitrogen; 34.72 mg L^{-1} phosphorus and 16.78 g L^{-1} carbon for maximum dry cell weight (4.88 g L^{-1}); 1.36 g L^{-1} nitrogen, 44.27 mg L^{-1} phosphorus and 17.35 g L^{-1} carbon for maximum lipid content (31.13 % wt.) and 1.37 g L^{-1} nitrogen, 59.03 mg L^{-1} phosphorus and 19.35 g L^{-1} carbon for maximum starch content (39.93 % wt.).

The aim of the present study was to maximize biomass, lipid accumulation and starch production by using the composite desirability function approach. The determination of optimal conditions for multi-response optimization is more informative and useful as compared to single response optimization [17]. Among the multi-response optimization, desirability function approach is one of the popular and recognized technique for determination of optimum setting of input variables and to determine optimum performance levels for one or more responses [29].

Fig. 4 shows the desirability ramp of the optimal solution for multi-response optimization. The maximum predicted response values for multi-response optimization were obtained to be 4.81 g L^{-1} (dry cell weight), 30.58 (% wt.) (lipid content) and 33.72 (% wt.) starch content at the optimum level of nitrogen, 1.93 g L^{-1}, 37.72 mg L^{-1} phosphorus and 17.27 g L^{-1} carbon. The composite desirability value to reach the optimum was 0.985, which fulfils 98.5% of the maximum obtainable response for each variable (Fig. 5). The comparative valuations of the combined effect of medium constituents on biomass-, lipid- and starch productivity from individual and multi-response optimized medium are summarized in Table 4. The maximum lipid productivity for individual and multi-response optimization was calculated at 160 and 210 mg L^{-1} d^{-1} respectively, suggesting an increase of 31.25% in multi-response than individual response optimization. Similarly, the maximum starch productivity for individual and multi-response optimization was calculated at 162.18 and 232.00 mg L^{-1} d^{-1} respectively and observed 43.05% improvement in starch productivity by multi-response when compared to individual response optimization.

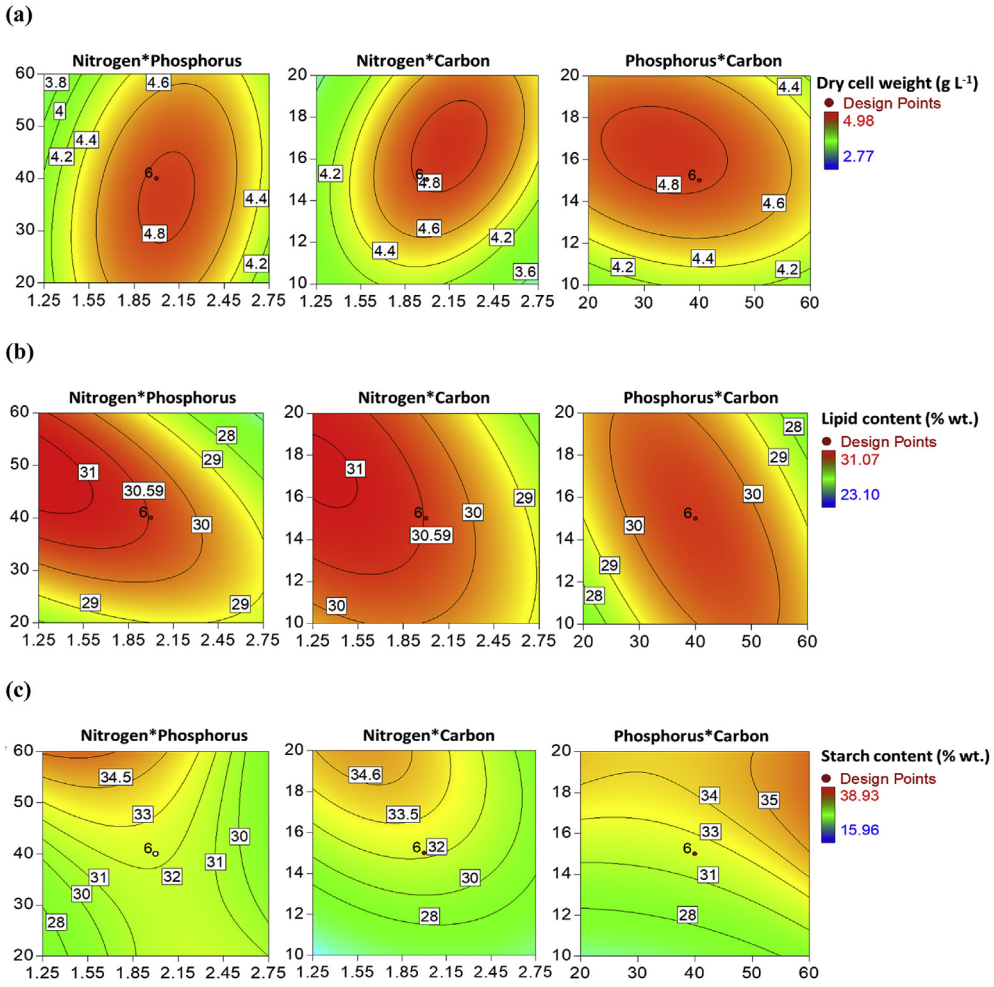

Fig. 2. Contour diagram for response (a) dry cell weight (gL^{-1}), (b) lipid content (% wt.) and (c) starch content (% wt.) with varying two variables at a time while the third variable was kept at its central value. Central values of variables were nitrogen (KNO_3) –2.0 g L^{-1}; phosphorus (anhy. K_2HPO_4)- 40 mg L^{-1}; carbon (glucose) - 15 g L^{-1} (Axis titles are given on the top of each figure and before asterisk represent X-axis title whereas after asterisk represent Y axis).

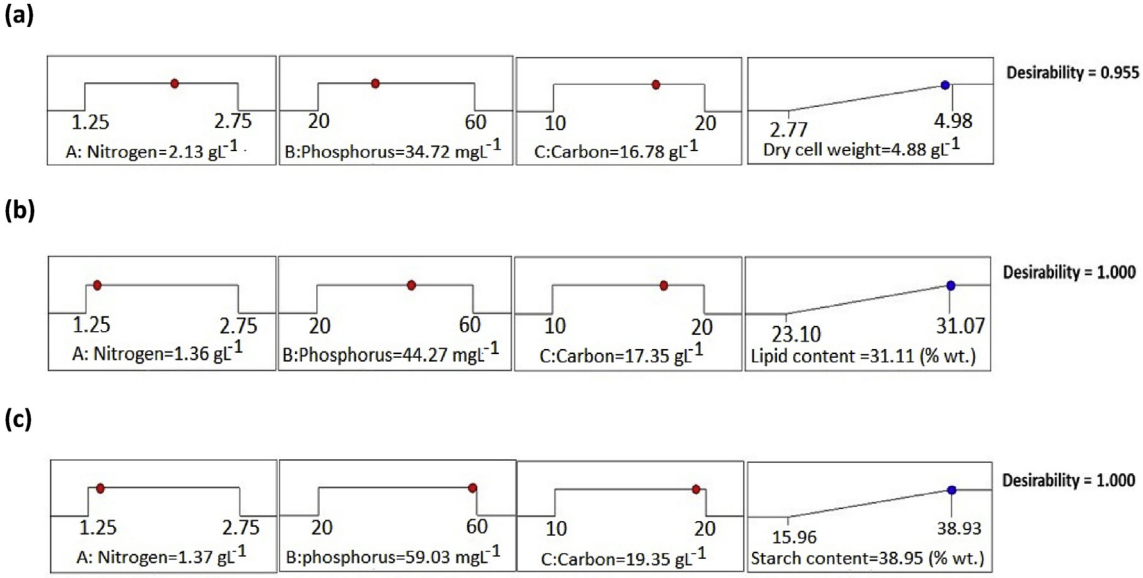

Fig. 3. The maximum response of (a) dry cell weight (g L-1), (b) lipid content (% wt.) and (c) starch content (% wt.).

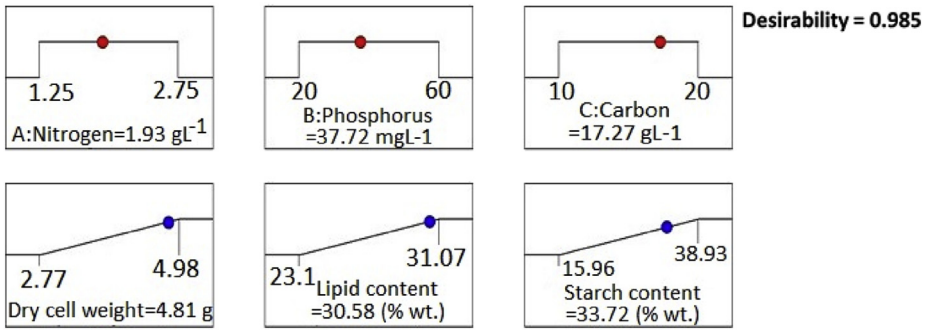

Fig. 4. Multi-response optimization condition to maximize the production of all three responses (dry cell weight, lipid content and starch content).

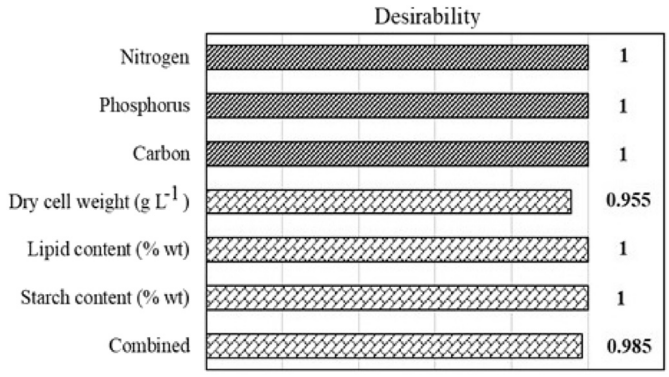

Fig. 5. Bar graph representing individual desirability of all responses (di) in correspondence with desirability.

3.6. Experimental verification of models

To confirm the developed models, the experiments were performed separately for all three responses (dry cell weight, lipid content and starch production) with modified BG-11 with their respective optimum levels of selected variables. *Scenedesmus* sp. ASK22 biomass was measured at every 24 h while the lipid content and starch production were measured at the end of 7 days batch cultivation. Growth, lipid content and starch production data obtained in original and modified BG-11 media is given in Fig. S3. The observed maximum dry cell weight (gL^{-1}), lipid content (% wt.) and starch content (% wt.) for *Scenedesmus* sp. ASK22 were 5.02 ± 0.10, 21.41 ± 0.67 and 24.98 ± 1.49, respectively. Maximum lipid accumulation (% wt.) 30.72 ± 0.23 was observed at dry cell weight (g L^{-1}) 3.18 ± 0.18 and starch production (% wt.) 31.60 ± 1.37. The observed maximum starch production was (% wt.) 37.10 ± 2.71 at 3.66 ± 0.17 dry cell weight (gL^{-1}) and 25.18 ± 1.32 lipid content (% wt). All these observed results are in very close agreement with the model's prediction (Fig. S4).

As shown in Table 5, the lipid productivity of *Scenedesmus* sp. ASK22 in multi-response optimized media (210.13 mg l^{-1} d^{-1}) was higher than reported values for production in various green microalgae [17,44–46]. Kanaga et al. [17] optimized the heterotrophic cultivation of *Chlorella pyrenoidosa* NCIM 2738 to enhance biomass, lipid and glucose consumption simultaneously and observed 1.30 g L^{-1} biomass and 30.61 mg $l^{-1}d^{-1}$ lipid productivity after 12-day batch cultivation. In another study it was reported that the carbon and nitrogen sources significantly influence the biomass and lipid accumulation of *Chlorella saccharophila*. Manipulation of these variables produced 7.7- fold higher biomass with 3-fold rise in lipid content in heterotrophic cultivation as compared to autotrophic cultivation at the optimum value of glucose, glycerol and bacterial peptone 40 g L^{-1}, 5 g L^{-1} and 1 g L^{-1}respectively [45]. Mohan and Devi, [46], proposed dual-mode cultivation (8 days biomass growth phase (BGP) followed by 7 days salinity induced lipid accumulation phase (LAP) of microalgal consortium collected from domestic effluent and achieved 408 mg $l^{-1}d^{-1}$ and 95.47 mg $l^{-1}d^{-1}$, biomass- and lipid productivity at the end of LAP (15 days). Zhu et al. [47] investigated the effect of nitrogen starvation on carbohydrate/starch accumulation in *Chlorella Zofingiensis*. The results suggested that the nitrogen starvation, quickly induced starch accumulation, and obtained 268 mg $L^{-1}d^{-1}$ starch productivity after 5 days cultivation, which is slightly higher than *Scenedesmus* sp. ASK22 starch productivity (231.70 mg $l^{-1}d^{-1}$). In a recent report, Cheng et al. [48] proposed a two-stage cultivation process for improving carbohydrate/starch production in *Chlorella* sp. AE10. First 3-days were selected to obtain enough biomass and 5–6 days were selected for starch accumulation, in the integrated

Table 4
Experimental verification of combined effect of optimized medium constituents on the response (dry cell weight (g L^{-1}), lipid- (% wt.) and starch content (% wt.) after 7 days batch cultivation.

Conditions	Nitrogen[a] (g L^{-1})	Phosphorus (K_2HPO_4) (mg L^{-1})	Carbon (Glucose) (g L^{-1})	Dry cell weight[b]	Lipid content[b]	Starch content[b]	Productivity[b] (mg $L^{-1}d^{-1}$)			Improvements in productivities (fold)		
							Biomass	Lipid	Starch	Biomass	Lipid	Starch
Unoptimized_BG11	1.50	40.00	–	0.381	16.42	10.79	63.50	10.43	6.85	–	–	–
After optimization												
Biomass production	2.13	34.72	16.78	5.02	21.41	24.98	717.14	153.54	179.14	11.29	14.72	26.15
Lipid content	1.36	44.27	17.35	3.18	30.72	31.60	454.29	139.56	143.56	7.15	13.38	20.96
Starch content	1.37	59.03	19.35	3.66	25.18	37.46	522.86	131.66	195.86	8.23	12.62	28.59
Multi-objective optimization	1.93	37.72	17.27	4.67	28.46	32.83	667.14	189.87	219.02	10.51	18.20	31.97

[a] $NaNO_3$: for control BG11 medium and urea: for individual and multi-response optimized media.
[b] All observed values of responses are mean values of duplicates and standard deviation are less than 3%.

Table 5
Comparison with other investigators.

Microalgae strain	Cell density (g L^{-1})	Lipid content (% wt.)	Starch/carbohydrate content (% wt.)	Biomass productivity ($mgL^{-1}d^{-1}$)	Lipid productivity ($mgL^{-1}d^{-1}$)	Starch productivity ($mgL^{-1}d^{-1}$)	References
C. pyrenoidosa NCIM 2738	1.30	28.90	–	105.90	30.60	–	[17]
Chlorella sp.	4.48	25.10	–	–	112.40	–	[44]
Chlorella saccharophila UTEX 247	1.10	37.00	–	–	58.50	–	[45]
Chlorella sp. AE 10	–	–	60.50/77.60	–	–	311.00/421.00	[48]
Scenedemsus quadricauda	3.39	22.10	–	–	107.10	–	[37]
Scenedesmus sp. R-16	3.46	43.40	–	–	250.27	–	[35]
Microalgae consortium	6.12	23.4	–	408.00	95.47	–	[46]
Chlorella zofingiensis	–	–	–	699.00	–	268.00	[47]
***Scenedesmus* sp. ASK22**	**4.67**	**28.46**	**32.83/-**	**667.14**	**189.87**	**219.02**	**Present study**

effect of limited nitrogen concentration (0.375 g L^{-1}), high light intensity (1000 μmol $s^{-1}m^{-2}$) and CO_2 concentration (10% v/v) and achieved 77.6 (% wt.) and 60.5 (% wt.) of carbohydrate and starch respectively at the end of day 5. The starch productivity was 311 mg $l^{-1}d^{-1}$ in day 6. All these observed results are in very close agreement with the results of present study.

In all the individual and multi-response optimized conditions the *Scenedesmus* sp. ASK22 accumulated more starch as compared to lipid. This may be better explained by the Morales-Sánchez et al. findings at the molecular level [49]. They performed a comparison of proteomes under the conditions that promote lipid/starch accumulation from unicellular green microalgae *Neochloris oleoabunadns* and concluded that short duration of nitrogen limitation up-regulates the carbohydrate synthesis related enzymes such as UDP-glucose-pyro-phosphorylase and starch synthase, that promote the accumulation of starch more prominently. However, under prolonged nitrogen starvation pyruvate dehydrogenase (involved in lipid biosynthesis) was over expressed. Besides this, an enzyme ADP glucose pyro-phosphorylase was also up-regulated, which hydrolyses the starch chain to channel the carbon flow towards lipid synthesis. Additionally, the dehydrogenases from the pentose phosphate pathways were up-regulated to supply reducing power in the form of NADPH for lipid synthesis and inorganic nitrogen assimilation. The heterotrophic cultivation favours *Scenedesmus* sp. ASK22 biomass-, lipid- and starch productivity as compared to autotrophic cultivation.

3.7. Fatty acid methyl ester analysis

The fatty acid methyl ester profile of *Scenedesmus* sp. ASK22 grown in standard BG11 (autotrophic) and BG11 optimized (heterotrophic) for lipid- and starch productivity was analysed by using GC-MS (Fig. 6). In fatty acid profile a predominance of palmitic (C16:0) and linoleic (C18:2) acid can be observed, along with a considerable amount of steric (C18:0) and linolenic (C18:3) acids. However, a predominance of palmitic (C16:0) and oleic (C18:1) acids can be observed, along with considerable amounts of stearic (C18:0) and linoleic (C18:2) acids in multi-response optimized medium. In the autotrophically grown culture, the unsaturated fatty acids are more prominent (~78.8%) than saturated fatty acids (~21.2%) as compared to a photoheterotrophically grown culture that holds around 63.93% unsaturated fatty acids and 36.07% saturated fatty acids. Fatty acids composition of culture grown photoheterotrophically suits more for bio-diesel because bio-diesel derived from the saturated fatty acids is stable whereas the unsaturated fatty acids give it more fluidity at low temperature [50,51]. In addition, it is also reported that biodiesel composition

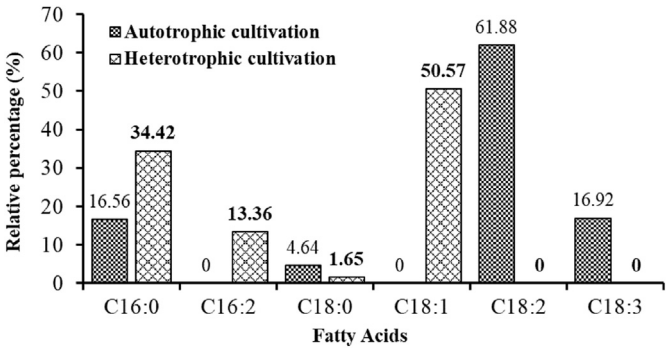

Fig. 6. Fatty acid profile of Scenedesmus spp. ASK22 grown in auto- and heterotrophic conditions.

enriched with C16–C18 fatty acids have good fuel properties [52].

4. Conclusion

The *Scenedesmus* sp. ASK22 grew effectively in modified BG11 medium and like glucose and urea as carbon and nitrogen sources respectively. The multi-response optimized heterotrophic metabolism of *Scenedesmus* sp. ASK22 gives 10.51-, 18.20- and 31.97-fold increase in biomass-, lipid- and starch productivity respectively as compared to autotrophic metabolism. The high lipid- and starch productivity in multi-response optimized medium proposed *Scenedesmus* sp. ASK22 as a suitable biofuel (biodiesel and bioethanol) feedstock. In addition, the presence of palmitic acid (16:0), stearic acid (18:0) and oleic acid (18:1) in multi-response optimized heterotrophic metabolism also proposed the *Scenedesmus* sp. ASK22 as a suitable biodiesel feedstock.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.renene.2019.12.095.

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