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Optimizing the Critical Factors for Lipid Productivity during Stress Phased Heterotrophic Microalgae Cultivation

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Microalgae-derived biodiesel production is one of the promising and sustainable platform. The effect of selected stress factors (pH, temperature, salinity, and carbon supplementation) on microalgal lipids and carbohydrate production during heterotrophic mode of operation was studied using design of experimental (DOE) methodology (Taguchi approach) with variation at four levels ($2^1 \times 4^4$). Experiments were performed with allegorical batch experimental matrix (16 experimental trails). All the selected factors showed marked influence on the lipid production, whereas temperature and carbon concentration showed major influence on the carbohydrate synthesis. Interesting, relatively higher total lipid production (55% of DCW) was obtained from Experimental no. 6 (pH: 6; salinity: 1 g/l; temperature: 20°C; carbon concentration: 30 g/l). Relatively good neutral lipid fraction (13.6%) was observed with Experimental no. 8: pH: 6; salinity: 5 g/l; temperature: 30°C; carbon concentration: 1 g/l. Good carbohydrate synthesis (262 mg/g biomass) was observed with Experiment no. 3 (pH: 4; salinity: 2 g/l; temperature: 30°C; carbon concentration: 15 g/l). Fatty acid methyl esters (FAME) analysis the presence of higher number of saturated fatty acids (C12:0 to C24:0) in experimental setups 6 and 8, favoring the biodiesel properties.

Keywords: biomass, chlorophyll, mixotrophic, nutritional mode, biodiesel, FAME, data envelopment analysis (DEA)

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INTRODUCTION

Microalgae cultivation is attracting renewed interest due to its ability to produce diverse photosynthetic products, including lipids. Microalgae can be cultivated either by autotrophic mode (Dayananda et al., 2007; Liu et al., 2011; Venkata Mohan et al., 2011) fixing CO_2 in the presence of sunlight or by heterotrophic mode (Brennan and Owende, 2010; Garcia et al., 2011; Devi and Venkata Mohan, 2012) using organic compounds as energy and carbon sources or by mixotrophic mode using both organic compounds and inorganic CO_2 (Qiao and Wang, 2009; Chen et al., 2011; Devi et al., 2012; Cong and Lu-Kwang, 2014; Cong et al., 2016). At present, inherent limitations encountered with microalgae cultivation is its low lipid/biomass production, which invariably impedes its scale-up operation.

To enhance lipid synthesis, strategies by altering the nutrient regime and cultivation conditions are generally employed. Factors, such as nutrient stress, light, temperature, CO_2, and salinity, have been explored to enhance lipid accumulation in microalgae (Merchant et al., 2012; Sharma et al., 2012; Chandra et al., 2014; Venkata Mohan et al., 2015; Chiranjeevi and Venkata Mohan, 2016). Stress has a critical role on the lipid synthesis during microalgae cultivation. The nutrient stress affects

the growth of algae as well as lipid productivity and its profile. Under adverse/stress conditions, microalgae tend to accumulate neutral lipids to protect cells from photooxidation (Adams et al., 2013; Zhang et al., 2013). Among the different modes of cultivation, heterotrophic operation offers several advantages over the others, including the elimination of light requirement, and lipid yields (Devi et al., 2012; Venkata Mohan et al., 2015). Moreover, in heterotrophic mode of operation, microalgae are influenced by the stress factors facilitating synthesis of higher lipid along with significant substrate degradation (Devi et al., 2012; Cong et al., 2016).

In order to study the effect of heterotrophically induced nutrient stress on the process, design of experimental (DOE) methodology was employed. The focal objective of this investigation is to study the methodological application of Taguchi orthogonal array (OA) experimental design (DOE) to optimize selected stress factors, viz., pH, salinity, temperature, and carbon supplementation. Factorial-based DOE methodology by Taguchi OA approach merges statistical and engineering techniques (Taguchi, 1986; Venkata Mohan et al., 2005, 2007). Mixotrophic microalgae cultivation was studied to achieve higher biomass productivity using Taguchi DOE methodology by optimizing eight factor (Chiranjeevi and Venkata Mohan, 2016).

Analysis of the experimental data using analysis of variance (ANOVA) provides information about statistically significant factors and their optimum levels.

EXPERIMENTAL METHODOLOGY

Design of Experimental Methodology

Taguchi's DOE methodology was used by selecting important factors, viz., pH, salinity, temperature, and carbon concentration, whose variation will have a critical effect on the heterotrophic lipid synthesis and carbohydrates production (**Table 1**). Four levels of factor variations were selected, which represent the experimentation size of 16 with an array matrix of M-16 (**Table 2**).

Microalgae

Mixed microalgae culture collected from Nacharam Lake (Pedda Cheruvu), Hyderabad, during the pre-monsoon season was used as parent inoculum. The culture was washed twice with water and pelletized (2.1 g with a concentration of 0.2 g/l) by centrifugation (3000 rpm; 10 min at 30°C) to remove associated debris and restored in rectangular plastic tubs (36 cm × 24 cm × 12 cm) exposed to diffused sunlight. Domestic sewage [(DS) pH, 7.8; COD, 220 mg/l; VFA, 165 mg/l; BOD, 120 mg/l; total alkalinity, 140 mg/l; chlorides, 175 mg/l; nitrates, 115 mg/l] was used as feedstock for microalgal cultivation. After the consistent amount of biomass (cell density) was achieved, this culture was used as inoculum for the experimental study.

TABLE 1 | Selected factors and assigned levels.

S. no.	Factor	Level 1	Level 2	Level 3	Level 4
1	pH	4	6	8	10
2	Salinity (g NaCl/l)	0	1	2	5
3	Temperature (°C)	20	25	30	35
4	Carbon supplementation (g glucose/l)	0	1	15	30

Experimental Details

The combinatorial of 4 factors at 4 levels (**Table 1**) with 16 experimental variations (M-16) in batch mode (**Table 2**), during stress phase (SP) was performed. In accordance with the designed experiments, the 250 ml of modified growth medium (as per design, **Table 1**) was sterilized (autoclaved for 20 min at 121°C and 1.05 kg/cm steam pressure) prior to the inoculation (10% v/v; OD, 0.1) to avoid the contamination. Growth phase (GP) was operated for a period of 8 days in mixotrophic mode [BG11 media, pH: 8.2, temperature: 32°C, glucose 1.5 g/l and light 4000 lux]. After 8 days of growth, the cultures were harvested, and the resulting biomass was used as inoculum for the SP of the experiments operated in heterotrophic mode (**Table 2**). All the experimental setups were placed on a temperature shaking incubator (120 rpm). During operation (GP and SP), biomass, pigment analysis (chlorophyll *a* and *b*) along with total carbohydrate concentration were estimated every alternate day. Lipid analysis was performed at initial (before the GP) and end of GP and SP. In both the phases of operations, to avoid bacterial contamination, antibiotic (ampicillin: 0.2 g/l) was added to each experimental setup once in every alternate day. All the experiments were carried out in triplicates, and the average of three independent operations was presented and discussed.

Analysis

Microalgae biomass was monitored by measuring OD at 600 nm and chlorophyll *a* and *b* were measured at 647 and 664 nm, respectively. Algal cells were disrupted by sonication (40 kHz for 2 minutes) followed by centrifugation (5000 rpm for 5 minutes at 28°C), and the dissolved carbohydrates in supernatant of the solution were estimated by Anthrone method. COD, nitrates, phosphates, and pH were analyzed as per standard procedure (APHA, 1998).

TABLE 2 | Orthogonal array (OA) of designed experiments with output parameters.

Experiment number	Factors				Lipid productivity (% of dry biomass)	Neutral lipid (%)	Total carbohydrates (mg/g biomass)
	1	2	3	4			
1	1	1	1	1	14.8	4.7	144
2	1	2	2	2	25.6	12.3	28
3	1	3	3	3	22.1	11.3	262
4	1	4	4	4	17.7	4.2	40
5	2	1	2	3	49.1	10.9	28
6	2	2	1	4	55.0	9.4	146
7	2	3	4	1	10.6	4.3	28
8	2	4	3	2	34.5	13.6	123
9	3	1	3	4	45.1	5.7	235
10	3	2	4	3	32.9	10.5	28
11	3	3	1	2	20.6	5.4	157
12	3	4	2	1	14.6	7	28
13	4	1	4	2	23.2	6.2	28
14	4	2	3	1	23.5	12.9	192
15	4	3	2	4	20.5	10	36
16	4	4	1	3	16	9.1	188

Lipid Extraction and Derivatization of FAME

At the end of GP (2.28 g/l) and SP (1.25–10.38 g/l), the biomass was separated (5000 rpm; 5 min; 28°C), and the resulting biomass after solar drying was powdered by blending. The blended powder was further sonicated (4 kHz; 2 min), and lipids was extracted using chloroform and methanol (2:1) as solvents (modified Bligh and Dyer method). Hexane was used for neutral lipid extraction (Devi et al., 2012). Furthermore, followed by centrifugation, lipid layer was transferred to pre-weighed round bottom flask, and the total and neutral lipids were determined gravimetrically in terms of percentage dry cell weight (% DCW). Lipid productivity (%) was calculated based on the ratio of total lipid extracted to dry weight of algal biomass. The resulted lipid was transesterified (using a methanol–sulfuric acid mixture) to fatty acid methyl esters (FAME). FAME composition was detected by gas chromatography (Nucon-5765) using FID with capillary column [30 mm (0.25 mm × 0.25 μm); Valcobond] using nitrogen carrier gas (1 ml/min). The oven temperature was maintained initially at 140°C (for 5 min), later increased to 240°C (ramp rate of 4°C/min; 10 min). The temperature of injector and detector were maintained at 280 and 300°C, respectively, with a split ratio of 1:10. The FAME composition was compared with the standard (C4- C24:1) (SUPELCO).

Data Analysis

The data derived from the experiments was analyzed employs "bigger is better" performance characteristics using Qualitek-4 (Nutek Inc.) software.

RESULTS AND DISCUSSION

Growth Phase

Mixotrophic mode of cultivation was used for microalgae cultivation during GP. Good increment in biomass growth [1.2 g/l (0 days) to 2.28 g/l with biomass productivity of 28.5 mg/l/day (8th day)] and marginal improvement in lipid productivity [total/neutral lipids 7.8/1.5 to 12.6/4.4% (8th day)] was observed. In agreement to the biomass growth pattern, the total chlorophyll (TC) also increased linearly from 1.3 to 23.6 μg/ml by the end of GP. The organic fraction (carbon) and nutrients (nitrogen and phosphate) contributed to the accumulation of carbohydrates (total, 40 mg/g biomass) and proteins (5.3 mg/ml) at the end of the GP operation.

Stress Phase

Selected Factors Influence on Lipid Synthesis

The influence of the selected factors on stress-induced heterotrophic microalgae cultivation showed marked effect on the lipid synthesis and profile. The magnitude of the difference between average effects (L2 − L1) represents the relative influence of the selected factors on the process. The average effect of the factors and resulting interaction on the total and neutral lipids synthesis is depicted in **Tables 3**–**5**. The bigger the difference, the stronger is the influence (ignoring the negative value). The experiments output showed a marked variation in the lipid production as well as on its profile. Among the four factors, pH showed a stronger effect on the total lipid synthesis, while temperature showed the least influence (pH > carbon supplementation > salinity > temperature). On the contrary, salinity showed a stronger effect on the neutral lipids synthesis, while pH showed least influence (salinity > temperature > carbon supplementation > pH).

TABLE 3 | Main effects of selected factors on total lipids production.

S. no.	Factor	Level 1	Level 2	Level 3	Level 4	L2 – L1
1	pH	20.049	37.299	28.299	20.799	17.25
2	Salinity (NaCl g/l)	33.049	34.25	18.45	20.7	1.201
3	Temperature (°C)	26.6	27.449	31.299	21.1	0.849
4	Carbon supplement (glucose g/l)	15.875	25.974	30.024	34.574	10.099

TABLE 4 | Main effects of selected factors on neutral lipids synthesis.

S. no.	Factor	Level 1	Level 2	Level 3	Level 4	L2 – L1
1	pH	8.125	9.549	7.15	9.549	1.423
2	Salinity (NaCl g/l)	6.875	11.274	7.75	8.475	4.398
3	Temperature (°C)	7.15	10.05	10.875	6.3	2.9
4	Carbon supplement (glucose g/l)	7.224	9.375	10.45	7.324	2.15

TABLE 5 | Main effects of selected factors on carbohydrates production.

S. no.	Factor	Level 1	Level 2	Level 3	Level 4	L2 – L1
1	pH	1.185	0.812	1.12	1.11	–0.373
2	Salinity (NaCl g/l)	1.087	0.982	1.207	0.947	–0.102
3	Temperature (°C)	1.587	0.3	2.029	0.31	–1.297
4	Carbon supplement (glucose g/l)	0.98	0.839	1.264	1.142	–0.142

The effect of pH on the total lipid synthesis was significant. Acidic redox microenvironment (pH 6) illustrated higher lipid synthesis. The favorable pH range for microalgae cultivation varies between 6 and 9, depending on the metabolic mode of cultivation and strain used. Heterotrophic mode cultivation between pH 6 and 7 was reported to be optimum (Kumar et al., 2010). The CO_2/organic carbon uptake by algae associates with an increase in biomass yield but leads to a decrease in pH that affects the microalgae physiology toward lipid synthesis (Belkin and Boussiba, 1991). Experiment no. 6 (pH 6, 30 g COD/l, 2 g NaCl/l, and 20°C) documented relatively higher lipid (total) productivity (55% of DCW; 21.62 mg/l/day) (**Figure 1A**). During cultivation of microalgae, the decrement in the pH from 6 to 4.8 was noticed. Acidic pH facilitates the diffusion of protons required for activation of hexose/proton symporter system for transport of glucose molecules from ambient cell surface to internal cytoplasm to take part in glycolysis pathway and subsequent lipid synthesis (Morales-Sánchez et al., 2013). With this experimental condition, protein and carbohydrates concentrations of 9.4 mg/ml and 146 mg/g DCW were observed, respectively. Subsequent to pH, carbon concentration (glucose) showed significant control on the

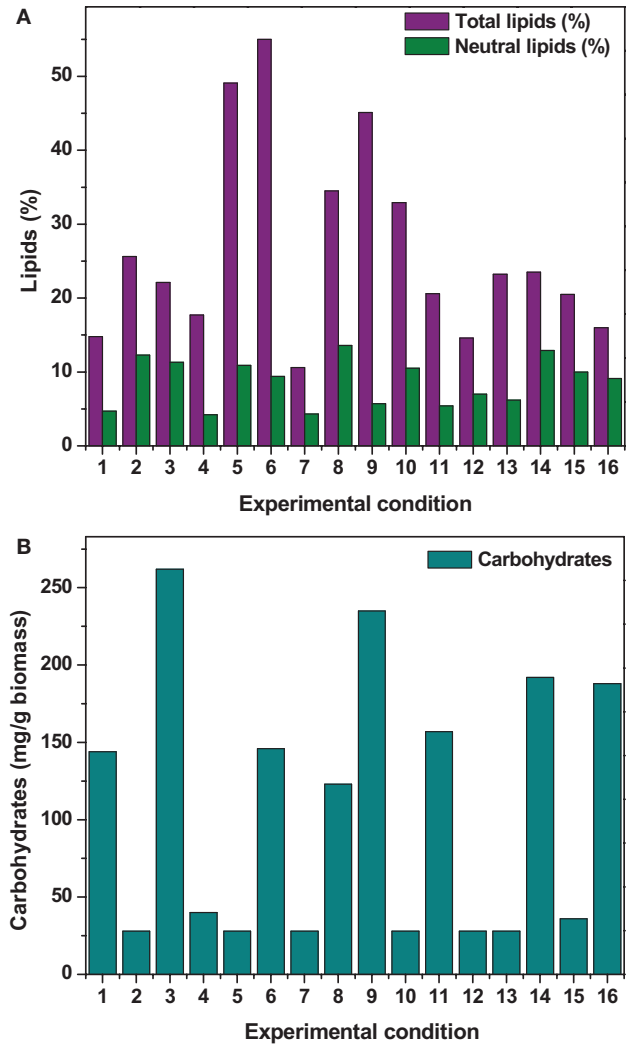

FIGURE 1 | **(A)** Total lipid and neutral lipid **(B)** carbohydrates concentration obtained from all the experimental conditions.

total lipid synthesis. Carbon in the form of glucose influenced the lipid accumulation (up to certain concentration). Glucose loading of 30 g/l at level 4 showed higher lipid productivity (55% of DCW; Exp no. 6). Carbon at optimal concentration during the SP increased the metabolic rate, manifesting glycolysis which also might influence the lipid synthesis (Chandra et al., 2014). Maximum biomass productivity (39.37 mg/l/day) with COD removal efficiency of 66% was observed with this experimental variation. Glucose absorption consumes less energy compared to CO_2 uptake by photosynthesis (Garcia et al., 2011). TC concentration [9.19 μg/ml (*chl a*, 6.61 μg/ml; *chl b*, 2.58 μg/ml)] also correlated with the biomass production.

Contrary to the total lipids, salinity (5 g/l NaCl) showed marked effect on the neutral lipids production [13.6% of DCW (total, 36% of DCW)] as documented with Exp no. 8 (1 g COD/l; pH 6; 30°C). Salinity-induced stress influences both physiological and biochemical mechanisms associated with the lipid synthesis (Kalita et al., 2011). Exposure of microalgae to hyper saline conditions alter their metabolism to uptake and export ions through the cell membrane, and the resulting stress proteins accumulated facilitates increment in the lipids content to adapt to the extreme environment (Talebi et al., 2013). Lipid induction phase operated under salt stress showed production of higher number of saturated fatty acid (SFA) methyl esters with the improved fuel properties along with increased lipid production (Venkata Mohan and Devi, 2014). The temperature at level 1 (20°C) and level 3 (30°C) showed favorable lipid synthesis. Cultivation at 30°C registered noticeable improvement in the lipid production, especially neutral lipids. The change in the cultivation temperature from optimal range invariably effects the composition of membrane lipids to maintain the normal function of microalgae (Subhash et al., 2014). With Exp no. 8, the pH increased from 6 to 7.1 during cultivation with COD removal efficiency of 86.2% and biomass productivity of 11.43 mg/l/day, were neutral lipid productivity of 3.88 mg/l/day [TC, 8.13 μg/ml (*Chl a/Chl b*, 5.61/2.51 μg/ml)]. The protein and carbohydrates concentration was found to be 7.68 mg/ml and 106 mg/g DCW, respectively.

Lipids Profile

The increase in cultivation temperature resulted in increased distribution of lipids in the form of SFAs (Boussiba et al., 1987). Temperature-induced stress showed influence on increment in the neutral lipid content illustrating feasibility toward good biodiesel properties with higher SFAs to unsaturated fatty acids (USFA) ratio (Subhash et al., 2014). Similar trend was observed in the present experiment with the presence of stearic (C18:0) and palmitic (C16:0) acids (**Figure 2**). The presence of long-chain fatty acids from C12:0 to C20:0 were observed in the Exp no. 8 due to the combined effect of salinity and temperature. The concentrations of SFAs were high with palmitic acid (C16:0) (50.8%), margaric acid (C17:0) (2.1%), and stearic acid (C18:0) (10.1%) compared to USFA [linoleic acid (C18:2) (6.8%); linolenic acid (C18:3) (2.8%)]. Fatty acids, including palmitic (40.7%) and myristic acids (4.2%), are known to have potential antibacterial and antifungal activities. Modification of fatty acids composition is one of the mechanisms used by algae to regulate osmotic balance and maintain membrane fluidity under the altered conditions (Adams et al., 2013). Low temperature induces the synthesis of more USFA to speed up the lipids desaturation as a recompensation measure to maintain the cell membrane fluidity (Perez-Garcia et al., 2011). Operating at lower temperature gives rise to more intracellular molecular oxygen and consequently improves the activities of desaturases and elongases that are involved in the biosynthesis of USFA (Chen and Chen, 2006). The presence of long-chain fatty acids from C12:0 to C24:0 were also observed due to the combined effect of pH and high carbon content.

Factor Influence on Carbohydrates Accumulation

Among the studied stress factors, temperature showed marked effect on the carbohydrate accretion, while salinity showed the least influence (temperature > pH > carbon supplementation > salinity). Nutrient limitation facilitates the accumulation of carbohydrates due to distinct reduction in cell growth (Ball et al., 1990). Temperature at level 3 (30°C) showed high carbohydrate accretion (262 mg/g biomass; Exp no. 3; 4 pH, 2 g

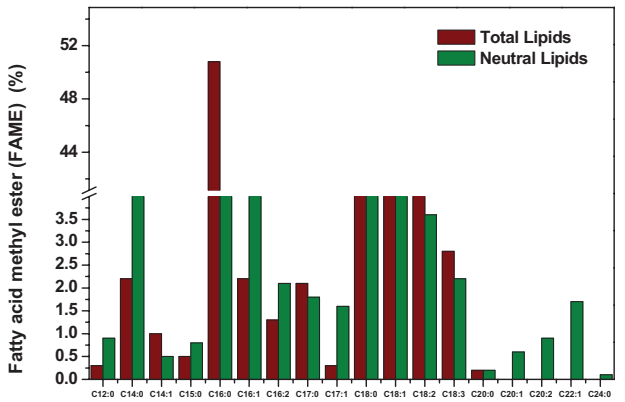

FIGURE 2 | Fatty acid methyl ester (FAME) composition of experimental condition six (TL) and eighth (NL).

NaCl/l; 15 g COD/l) than level 1 (20°C), level 2 (25°C), and level 4 (35°C) (**Table 2**; **Figure 1B**). Next to the temperature, carbon load (level 3; 15 g COD/l) showed feasibility to higher accretion of carbohydrate with substrate degradation of 66%. Carbohydrate accumulation improves by increasing the carbon load during the SP of microalgae cultivation (Giordano, 2001; Xia and Gao, 2005; Chandra et al., 2014). Under nutrient limiting conditions and with good supply of carbon, the protein content of microalgae function as nitrogen source, and the carbohydrate content may increase considerably (Ball et al., 1990). Salinity at level 3 (2 g/l NaCl) showed good carbohydrates accumulation. Accumulation of carbohydrates takes place as a response to an immediate NaCl shock (Zheng et al., 1997). Salt stress facilitates production of reactive oxygen species, which inhibit RuBisCO activity and lead to photoinhibition that concurrently decreases the biomass growth (Neale and Melis, 1989; Murata et al., 2007). High NaCl concentration also inactivates the ATP-synthase resulting protein synthesis inhibition in microalgae (Allakhverdiev et al., 2005). With Exp no. 3, the pH varied marginally from 4 to 3.9 during cultivation with COD removal efficiency of 66% and biomass productivity of 19.0 mg/l/day [TC, 7.3 μg/ml (*Chl a/Chl b*, 5.61/1.69 μg/ml)]. The protein concentration was found to be 10.71 mg/ml.

Maximum substrate degradation (based on COD removal efficiency) of 66, 86, and 66% was observed at the end of SP in experiments 6, 8, and 3, respectively. Apart from the lipid synthesis, the substrate utilized by the microalgae also associates with the biomass/carbohydrate production (6.37/146, 1.83/123, and 3.9/262 g/l/mg/g) during the heterotrophic microalgae cultivation under starvation phase. Maximum chlorophyll concentration was observed at the end of the starvation phase (Exp no. 6: 9.19 μg/ml; Exp no. 8: 10.9 μg/ml; Exp no. 3: 7.3 μg/ml) along with good protein concentration (Exp no. 6: 9.46 mg/ml; Exp no. 8: 7.68 mg/ml; Exp no. 3: 10.71 mg/ml).

Factor Interactions

Evaluating factors' interaction facilitates understanding the complexity of the process. The estimated interactions based on the severity index (SI) help to know the influence of two individual factors at various levels (**Table 6**). Salinity with temperature showed highest SI (71.73%), followed by pH and temperature (18.8%), pH and carbon supplementation (14.75%) (**Figure 3**). Temperature showed high level of interaction with pH (71%). The interaction between carbon and temperature (SI, 5.8%) showed a marginal influence on carbohydrate production. Interaction between salinity and pH (SI, 5.5%) has no significant influence on lipid and carbohydrate production.

TABLE 6 | Automated test for presence of interaction factors.

S. no.	Interacting factor pairs (based on SI)	Columns	SI (%)	Reserved column	Optimum levels
1	Salinity (NaCl) × temperature (°C)	2 × 3	71.73	1	[2,1]
2	pH × temperature (°C)	1 × 3	18.8	2	[2,1]
3	pH × carbon supplement	1 × 4	14.75	5	[2,4]
4	Salinity (NaCl) × carbon supplement	2 × 4	7.09	6	[2,4]
5	Temperature (°C) × carbon supplement	3 × 4	5.85	7	[1,4]
6	pH × salinity (NaCl)	1 × 2	5.51	3	[2,2]

Analysis of Variance

It is evident from ANOVA that all the factors and interactions considered in the experimental design had statistically significant at 95% confidence limit (*F*-ratios). The experimental degree of freedom (DOF) is 15 (factors-DOF, 3) (**Tables 7–9**). Salinity had the maximum contribution (29.12%) on total lipid production, followed by pH (27.97%) and carbon supplementation (27.49%) at the individual level (**Table 7**) (**Figure 3A**). The temperature had minimal impact on the lipid synthesis (6.33%). On the whole, salinity, pH, and carbon supplementation at their individual levels contributed over 95% of the influence on the lipid production, indicating that these factors play crucial role on lipid synthesis. The temperature had the maximum contribution (32.82%) on neutral lipid synthesis at the individual level, followed by salinity (23.08%) and carbon supplementation (14.56%) (**Table 8**) (**Figure 3B**). pH showed minimal impact on the production process (5.84%). Overall, temperature, salinity, carbon supplementation, and pH at their individual levels contributed over 76.3% of the influenced factors in an escalation of neutral lipid synthesis. The temperature showed maximum contribution (85.25%), followed by carbon supplementation on carbohydrate accumulation at the individual level (**Table 9**) (**Figure 3C**). On the whole, temperature and carbon supplementation at their individual levels contributed over 86.013% of the influence on carbohydrates production.

Optimum Conditions for Maximum Productivity

Optimum conditions during SP of operation to achieve higher lipid (total) productivity from microalgae by the selected factors contribution is shown in **Table 10**. The optimum operating conditions

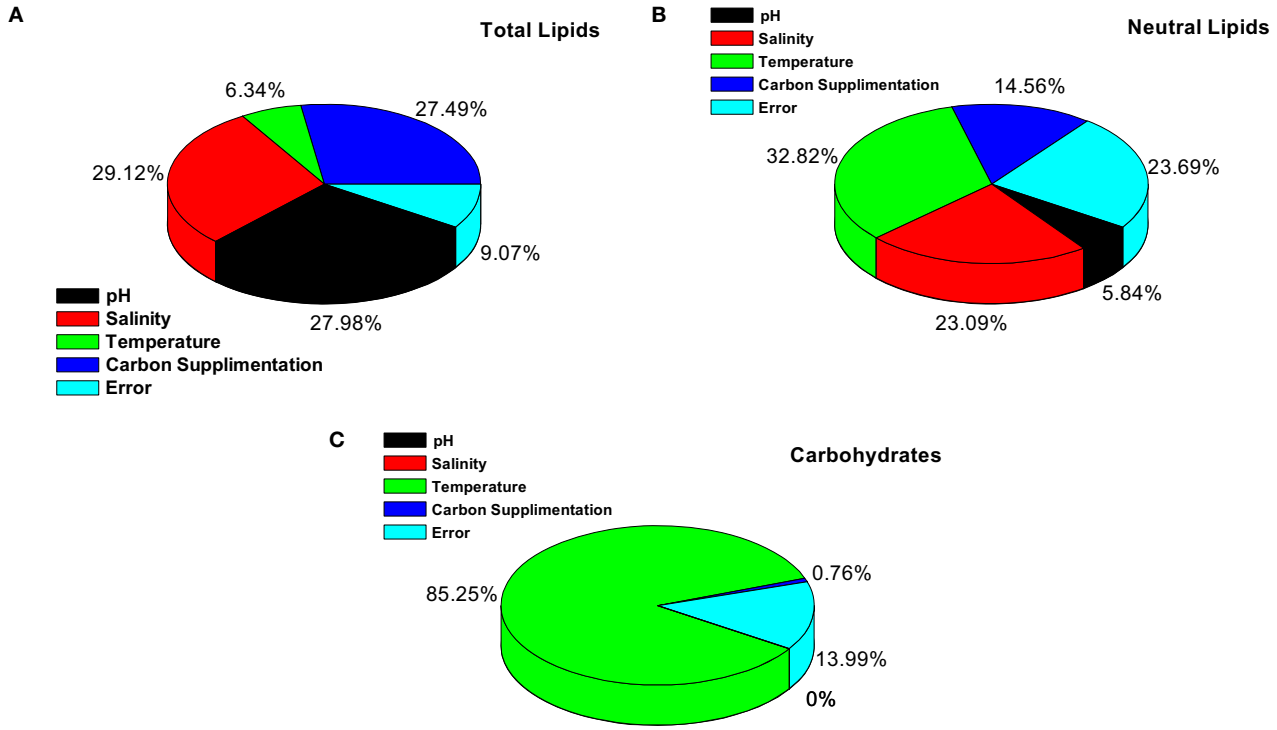

FIGURE 3 | **Significant factor and interaction influence on the output of (A) total lipids, (B) neutral lipids and (C) carbohydrates.**

TABLE 7 | Analysis of variance (ANOVA) for total lipids production.

S. no.	Factor	DOF	Sum of squares	Variance	*F*-ratio	Pure sum	Percent
1	pH	3	775.686	258.562	16.416	728.437	27.97
2	Salinity (NaCl g/l)	3	805.427	268.475	17.046	758.177	29.12
3	Temperature (°C)	3	212.247	70.749	4.492	164.997	6.337
4	Carbon supplement (glucose g/l)	3	762.986	254.328	16.147	715.736	27.49
	Other/error	3	47.249	15.749			9.075
	Total	15	2603.597				100

TABLE 8 | Analysis of variance analysis (ANOVA) for neutral lipids synthesis.

S. no.	Factor	DOF	Sum of squares	Variance	*F*-ratio	Pure sum	Percent
1	pH	3	16.513	5.51	2.233	9.129	5.843
2	Salinity (NaCl g/l)	3	43.476	14.492	5.873	36.074	23.088
3	Temperature (°C)	3	58.681	19.56	7.927	51.28	32.819
4	Carbon supplement (glucose g/l)	3	30.156	10.052	4.074	22.755	14.563
	Other/error	3	7.40	2.47			23.69
	Total	15	156.25				100

TABLE 9 | Analysis of variance (ANOVA) for carbohydrates production.

S. no.	Factor	DOF	Sum of squares	Variance	*F*-ratio	Pure sum	Percent
1	pH	3	0.331	0.11	0.996	0	0
2	Salinity (NaCl g/l)	3	0.163	0.054	0.489	0	0
3	Temperature (°C)	3	9.436	3.145	28.346	9.103	85.251
4	Carbon supplement (glucose g/l)	3	0.414	0.138	1.244	0.081	0.762
	Other/error	3	0.331	0.11			13.98
	Total	15	10.678				100

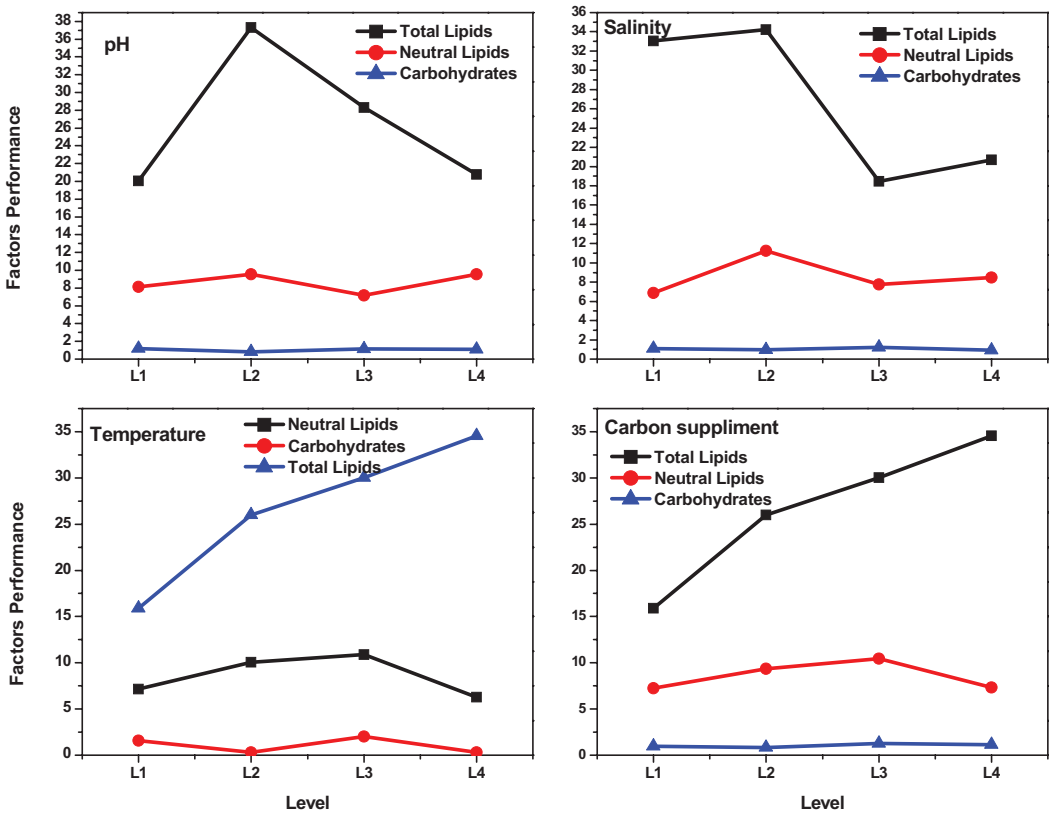

FIGURE 4 | Individual factors performance at different levels.

TABLE 10 | Optimum conditions and their contribution on total lipids.

S. no.	Factor	Level description	Level	Contribution
1	pH	6	2	10.687
2	Salinity (NaCl g/l)	1	2	7.637
3	Temperature (°C)	30	3	4.687
4	Carbon supplement (glucose g/l)	30	4	7.962
Total contribution from all factors				30.97
Current grand average of performance				26.61
Expected result at optimum condition				57.58

TABLE 11 | Optimum conditions and their contribution on neutral lipids.

S. no.	Factor	Level description	Level	Contribution
1	pH	6	2	0.956
2	Salinity (NaCl g/l)	1	2	2.681
3	Temperature (°C)	30	3	2.281
4	Carbon supplement (glucose g/l)	15	3	1.856
Total contribution from all factors				7.77
Current grand average of performance				8.59
Expected result at optimum condition				16.36

showed the requirement of acidic microenvironment (pH 6) with 1 g NaCl/l, 20°C, and 30 g COD/l, which resulted in the increment of lipid (total) production from 14.6 to 57.6% (from 26.6%) (**Figure 4**). A total contribution of factors at optimized conditions was 30.97% for lipid production with a maximum contribution of 10.68% from pH. Salinity was specifically found to be the most significant factor influencing neutral lipid production (**Table 11**). The optimum operating conditions showed the requirement of acidic microenvironment (pH 6) with 1 g NaCl/l, 30°C, and 15 g COD/l, which resulted in the increment of neutral lipid from 8.6% to 16.4% (**Figure 4**). The temperature was found to be the most important factor influencing carbohydrate production (**Table 12**).

TABLE 12 | Optimum conditions and their contribution on carbohydrates.

S. no.	Factor	Level description	Level	Contribution
1	pH	4	1	0.128
2	Salinity (NaCl g/l)	2	3	0.15
3	Temperature (°C)	30	3	0.973
4	Carbon supplement (glucose g/l)	15	3	0.208
Total contribution from all factors				145.9
Current grand average of performance				105.6
Expected result at optimum condition				251.5

The favorable operating conditions showed the requirement of acidic microenvironment (pH 4) with 2 g NaCl/l, 30°C, and 15 g COD/l results in the increment of carbohydrate production from 140 to 251.5 mg/g biomass (**Figure 4**). Carbohydrate profile showed the presence of 35% of xylose and 20% of arabinose.

DISCUSSION

Data envelopment analysis (DEA) technique was used to analyze the relative performance of the system based on the output parameters viz., lipid productivity (total and neutral) and carbohydrates production. DEA offers an easy approach to measure, compare the performance, and analyze the results obtained by interpreting the output of a given system based on the relative efficiency (not on absolute efficiency) using graphical approach (Venkata Mohan et al., 2008). The relative efficiency of any given system indicates the extent to which other systems can improve its performance. Total and neutral productivity and carbohydrates production were considered as three output parameters corresponding to input parameter (pH) (**Table 13**; **Figure 5**). The relative efficiency of the system can be calculated by comparing the current performance of the system to the best possible performance that the system could be reasonably expected to achieve as per the Eq. 1, where, X represents the length of the line from the origin to the point obtained by plotting two ratios of the system and Y denotes length of the line from the origin through the point obtained by the system to the efficient frontier.

$$\text{Relative efficiency} = [(X/Y) \times 100] \quad (1)$$

Experiment 6 (carbon concentration 30 g/l; temperature 20°C; salinity 1 g/l; pH 6), experiment 3 (carbon concentration 15 g/l; temperature 30°C; salinity 2 g/l; pH 4) and experiment 2 (carbon concentration 1 g/l; temperature 25°C; salinity 1 g/l; pH 4) yielded maximum efficiency (100%) among the experimental variations studied with respect to total lipids, neutral lipids and carbohydrates productivities. In all the three cases of data analyses by DEA considering pH as input, experimental points on the efficient frontier lines (relative efficiency 100%) illustrated the efficacy of acidophilic pH (6–4) with salinity (1 or 2 g/l), carbon supplemented with high glucose concentration and temperature in the range of 20–30°C. About six of the experimental variations studied are above 60% of the relative efficiency. Experiments above 96% relative efficiency showed positive influence of acidophilic cultivation.

TABLE 13 | Consolidated data of DEA analysis.

TL vs NL		TL vs Carbohydrates	
Experiment number	**Relative efficiency (%)**	**Experiment number**	**Relative efficiency (%)**
6	100	6	100
2	100	3	100
5	96.8	2	90.1
3	91.0	5	71.9
8	82.1	9	70.6
9	62.5	8	66.1

As shown in **Table 14**, microalgae cultivation at pH 6 increased lipid productivity with more number of SFAs in both experiments 6 and 8. Influence of salinity was observed markedly on increment in palmitic acid (C16:0) fraction. Microalgae can survive in extreme microenvironments; a minor shift in pH in external environment can change their metabolic activity (Varshney et al., 2015). A change in acidophilic environment from pH 6 to 4, microalgae showed transition of biochemical pathway from lipid to carbohydrates synthesis when experimental conditions 6/8 and 3 were compared. Studies also reveal that at acidophilic conditions showed increased basal level of heat shock protein (HSP), higher levels of the antioxidative enzymes, ascorbate peroxidase (APX) and which is thought to be an adaptive mechanism to extreme acidic environment (Gerloff-Elias et al., 2006; Garbayo et al., 2007). The acidophiles also showed increased production of the antioxidants lutein and β-carotene, respectively (Garbayo et al.,

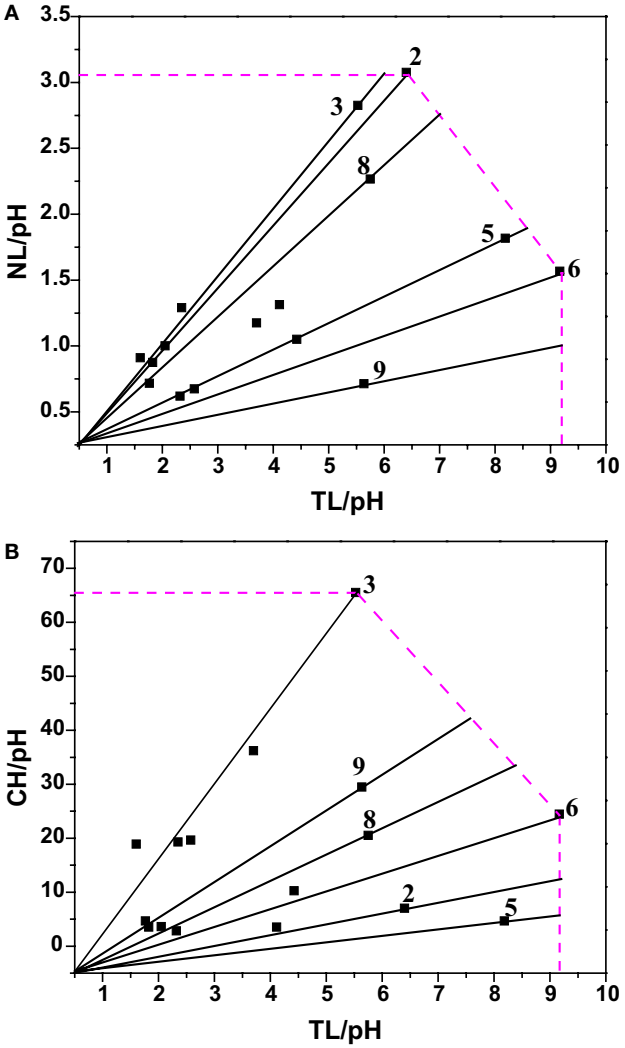

FIGURE 5 | Experimental data expressed in DEA format to calculate the relative efficiency [(A) Neutral Lipids (NL) rate and Total Lipids (TL) vis a vis pH; (B) Carbohydrate (CH) and Total Lipid (TL) vis a vis pH].

TABLE 14 | Consolidate optimum conditions toward biomass and lipid production obtained from the study.

Experiment number	Experimental condition				Total/neutral lipids (% DCW)	Biomass production (g/l)	Lipid productivity (mg/l/day)	SFAs	UFAs		Carbohydrates (mg/g biomass)
	pH	Salinity (g/l)	Temperature (°C)	Carbon supp (g/l)					MUFA	PUFA	
Experiment No. 6	6	1	20	30	55.0/9.4	6.3	21.62	7 No. (C16:0; 40.7%)	5 No. (C18:1; 21.4%)	4 No. (C18:2; 3.6%)	146
Experiment No. 8	6	5	30	1	34.5/13.6	1.8	3.88	6 No. (C16:0; 50.8%)	3 No. (C18:1; 19.4%)	3 No. (C18:2; 6.8%)	123
Experiment No. 3	4	2	30	15	22.1/11.3	3.9	4.21				262
Total lipids (**Table 10**)[a]	6	1	30	30	57.5						251
Neutral lipids (**Table 11**)[a]	6	1	30	15	16.3						
Carbohydrates (**Table 12**)[a]	4	2	30	15							

[a]*Optimum condition.*

2008; Vaquero et al., 2012). Increase in carbon concentration and salinity favored high number of mono unsaturated fatty acids (MUFA). In experiment No. 6, more number of polyunsaturated fatty acids (PUFAs; structural lipids) with major fractions of oleic acid (C18:1) and linoleic acid (C18:2) was observed, which basically contributes to the high biomass productivity. Higher lipid fraction (55% of DCW) and biomass productivity (39.3 mg/l/day) were obtained with high carbon, minimal salinity, and low temperature conditions (Experiment No. 6).

CONCLUSION

Design of experimental methodology based on Taguchi approach illustrated the specific function of selected factors on microalgae lipid synthesis and carbohydrate production during stress-induced heterotrophic cultivation. Among the four factors, pH, salinity, and carbon concentration showed significant influence on the lipid synthesis. This study reported a high total lipids concentration (55% of DCW; 21.6 mg/l/day), observed in high carbon, minimal salinity, and low temperature conditions. A maximum neutral lipids concentration (13.6% of DCW; 3.8 mg/l/day) was observed in high salinity, low carbon conditions at acidophilic microenvironment. Acidophilic microenvironment, moderate salinity, and high carbon concentration illustrated significant influence on the carbohydrates synthesis. The FAME analysis documented good biodiesel properties with a higher number of SFAs, especially palmitic acid (C16:0) followed by MUFA and PUFA with major fractions of oleic acid (C18:1) and linoleic acid (C18:2). The study documented feasibility of heterotrophic mode of microalgae cultivation toward regulating higher lipid productivity, along with waste treatment.

AUTHOR CONTRIBUTIONS

All the authors have equally contributed to the manuscript and mutually agreed for submission of the work.

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