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Effect of Salinity Stress on Lipid Accumulation in Scenedesmus sp. and Chlorella sp.: Feasibility of Stepwise Culturing

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Abstract

The enhanced lipid accumulation in microalgae is envisioned under special stress conditions with the cost of algal growth, which in turn affects the overall lipid productivity. The selection of suitable stress conditions facilitates better lipid productivity without any harmful effect on microalgae growth and algal biomass production. In the present study, we have attempted to select the best salinity conditions towards better growth, biomass accumulation, and lipid productivity of microalgae. The study also envisaged testing the feasibility of the stepwise salinity stress-induced cultivation approach to minimize the growth penalty effect of microalgae. The highest specific growth rate (0.129, 0.133, 0.113 μday^{-1}) and doubling per day (0.185, 0.193, 0.163 per day) were obtained at salinity concentration of 40 mM NaCl in BG-11 medium for *Scenedesmus quadricauda* (*Sq19*), *Scenedesmus dimorphus* (*Sd12*), and *Chlorella* sp. (*Chl16*), respectively. Maximal lipid content of 18.28, 30.70, and 32.19%, and lipid productivity of 8.59, 13.81, and 10.27 mg l^{-1} day^{-1} were achieved at 160 mM of NaCl in BG-11 media with the *Sq19, Sd12, and Chl16* algal isolates, respectively. The utilization of stepwise salinity stress (160 mM) induced cultivation of *Sd12* algal isolate results in higher lipid content (39.42%) and slightly improved lipid productivity than the control (without any stress, 20.4% lipid content). The results indicate the feasibility of enhancing the lipid content and productivity through the salinity-induced stepwise cultivation strategy.

Introduction

The energy crisis is one of the significant challenges being faced by the entire human race worldwide. Intensive research efforts are going on towards the development of alternative biomass-derived biofuels to substitute the depleting fossil fuels [1]. Among different biomass-based alternatives, the microalgal lipid is one of the promising feedstock for biodiesel production. The positive attributes coupled with the microalgae include the faster growth rate using wastewater resources with the sequestration of atmospheric CO2 and lipid accumulation potential without disturbing the food chain [2]. Microalgal lipids serve as a suitable substrate for biodiesel production due to the high energy content of the accumulated lipids. The energy density (41 MJ/Kg) of biodiesel from microalgae lipids is comparable to that of petroleum diesel (40–45 MJ/Kg) and superior to the biodiesel from plant oils (37.0 MJ/Kg) [3, 4]. Research so far suggests that the high lipid accumulation in the algal cells occurs under special stress conditions, which also negatively affect the growth and overall lipid productivity too [5, 6]. A minimal number of microalgal species have explored for lipid accumulation under stress conditions on a laboratory scale studies which pave the way for extrapolation of lab results to commercial scales.

The microalgal lipid production can be enhanced through the varying the cultivation conditions and subjecting to diverse stress conditions such as temperature [7], light intensity [8], salinity [9], nutrient stress [10], and mineral stress [11]. The proposed/adopting stress condition towards enhanced lipid production needs to be amenable to execute on a commercial scale perspective with the minimal effect of on algal growth. Stress-induced lipid production with minimal impact on algal growth also facilitates developing

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a tolerant strain in an open environment, which eventually results in a two-way reduction in the production cost. In our laboratory, we have screened native isolates of three dominant microalgae *Scenedesmus dimorphus*, *Scenedesmus quadricauda*, and *Chlorella* sp. and standardized the nitrogen stress on selected isolates intending to achieve higher growth and lipid productivity [12]. Another stress which seems comparatively easy and cost-effective to apply to grow algal cultures on a large-scale production environment is salinity stress. It is well documented that saline stress can lead to increment in the lipid content of microalgae [9, 11, 13]. It is believed under high salinity stress, lipid content and the proportion of saturated fatty acids increase compared to polyunsaturated fatty acids in a quest to adapt in an extreme environment [14]. Salinity stress also results in a higher amount of saturated fatty acids, which are desirable for ideal fuel properties. The addition of NaCl significantly affects biomass and growth and lipid accumulation in *Scenedesmus* sp. CCNM 1077 in a dose-dependent manner. The salinity stress induced by the addition of 400 mM NaCl for three days in stepwise cultivation mode resulted in enhanced lipid content with negligible biomass reduction [9]. Salinity manipulations and the use of salt-tolerant strains can be one of the strategies to reduce microbial contamination [13, 15]. The reported studies indicate the profound effect of induced salinity stress on the enhanced growth and lipid production of microalgae. Further studies are needed to optimize the favorable stress conditions to achieve enhanced lipid productivity with minimal effect on growth.

Hence, in the present study, we attempted to optimize salinity stress in the growth media for maximum growth, biomass accumulation, and lipid productivity. We also tested the feasibility of the stage cultivation and production approach to minimize the growth penalty effect of applied salinity stress.

Materials and Methods

Microalgal Isolates, Medium, and Experiment Conditions

The isolated and characterized microalgal isolates of our laboratory, i.e., *Scenedesmus quadricauda (Sq19)*, *Scenedesmus dimorphus (Sd12)*, and *Chlorella* sp. (*Chl16*), were used for the present study [12]. The stock cultures were maintained regularly on both agar plates and BG-11 liquid media. For a single-step salinity experiment, microalgal isolates were grown in BG-11 and CHU-10 media with different initial NaCl concentrations (40, 80, 160, and 320 mM) throughout the stationary phase (18 days) of the growth period. For a stepwise cultivation experiment, microalgae isolate (*Sd12*) was first grown in BG-11 medium (without any NaCl) and followed by the addition of 160 mM NaCl to the BG-11 media after 18 days of cultivation for inducing the salinity stress. All the experiments were carried out in triplicates (through individual experiments) using 1000-ml flasks containing 800 ml of culture medium inoculated with the respective 10% active microalgal isolate. The inoculated flask contents of microalgal isolated were grown at 18–25 °C using a 16:8-h light and dark cycles [16].

Determination of Growth and Biomass Productivity

Microalgal growth was monitored every 3rd day by measuring the OD (Optical Density) at 750 nm and cell counts using Neubauer chamber (0.25 mm depth). The maximum cell density, specific growth rate (μ), doubling per day (K), and doubling time (Tt) were calculated using the following equations [17]:

$$\mu = \ln\left(N_t/N_0\right)/T_t - T_0 \tag{1}$$

where N_t is number of cells at the end of log phase, N_0 is number of cells at the start of the log phase, T_t is final day of log phase, and T_0 is starting day of log phase.

$$K = \mu/0.6931 \tag{2}$$

$$T_t = 0.6931/\mu \tag{3}$$

The collected cultures were harvested by centrifugation at 7000 rpm for 10 min. After that, the pellet was freeze-dried at − 80 °C and lyophilized. The dry weight of microalgal biomass was determined gravimetrically and expressed dry weight obtained per liter (g L^{-1}) [18].

The biomass productivity (g L^{-1} day^{-1}) was calculated using the following equation:

$$B_p = T_b - I_b/F_d \tag{4}$$

where T_b is biomass achieved at stationary phase, I_b is biomass at starting of log phase, and F_d is no. of days at stationary phase. Biomass productivity (B_p) was expressed as the dry biomass produced in g L^{-1} day^{-1}.

Determination of Lipid Productivity

The total lipid from microalgal biomass was extracted by using the Bligh and Dyer method [19] with some modifications. The microalgal biomass (200 mg) was suspended in 5 ml of chloroform and 10 ml of methanol and mixed well. The suspension was subjected to sonication for 10 min with 10 s on and 5 s off pulse to accomplish the cell disruption. The lower chloroform layer from the formed biphasic layers was taken in a centrifuge tube and further purified with the 0.4 ml of distilled water. The obtained

lipid content was transferred to a clean centrifuge tube, and total lipids content (Lc, percent of dry biomass) was quantified and lipid productivity was calculated by using the following formula.

$$Lp = Bp \times Lc \times 1000/100 \quad (5)$$

where Bp is biomass productivity and Lc is percent lipid content.

Statistical Analysis

The significant difference among adopted algal cultivation parameters was studied through one-way analysis of variance (ANOVA). The standard error (SE) values were depicted in the graphs as error bars. The superscripts in the tables denote the rankings based on Tukey's HSD[a] multiple range tests, and different alphabets in tables indicate the significantly different values. The effect of interactions of varying cultivation parameters was drawn through the three-way analysis of covariance with contrasts through the general linear model (GLM) approach with the help of SPSS 17 software.

Results and Discussion

Effect of Salinity on Growth and Biomass Productivity

The isolates *Sq19, Sd12,* and *Chl16* were able to grow in all the tested concentrations of sodium chloride (40–320 mM). The optical density and cell counts decreased with increasing levels of sodium chloride in both BG-11 and CHU-10 medium. The maximum optical density (0.528, 0.847, and 0.766) and cell count (88.33, 97.27 and 98.28 cells ml^{-1}) with respect microalgae isolates *sq19*, *sd 12*, and *chl16,* respectively, were found at 40 mM among all tested concentrations in both media (Figs. 1, 2). The specific growth rate (μ) and doubling per day (k) of microalgal isolates *Sq19, Sd12,* and *Chl16* were found to be significant with the induced salinity concentration. The highest specific growth rate (0129, 0.133, 0.113, μday^{-1}) and doubling per day (0.185, 0.193, 0.163, per day) were observed with

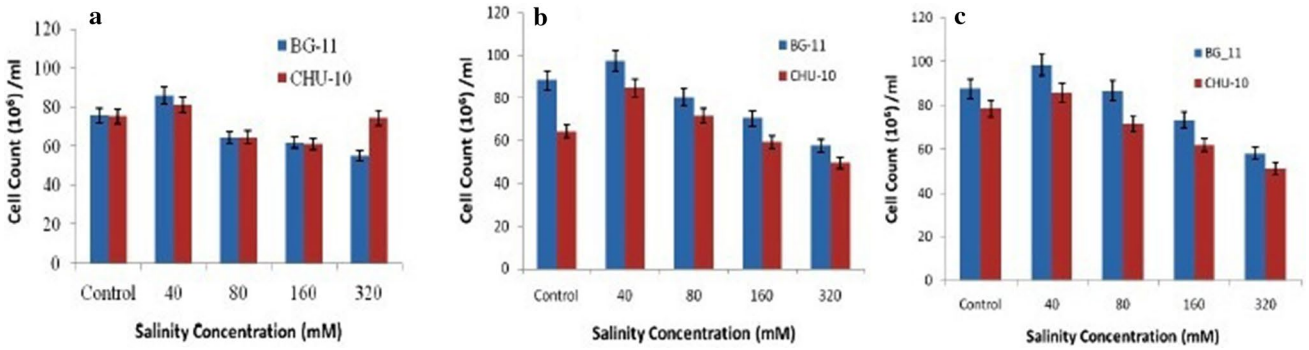

Fig. 1 Effect of different NaCl concentration on cell growth of *Sq19* (**a**), *Sd12* (**b**), and *Chl16* (**c**) isolates during single stage. Data are the mean values and display error bars for the selected chart series with 5% value

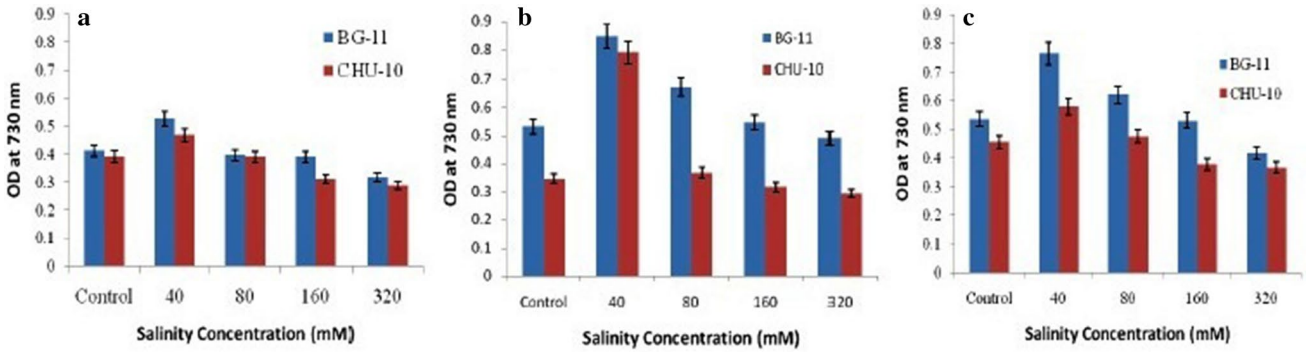

Fig. 2 Effect of different NaCl concentration on optical density of *Sq19* (**a**), *Sd12* (**b**), and *Chl16* (**c**) isolates during single stage. Data are the mean values and display error bars for the selected chart series with 5% value

the microalgal isolates, i.e., *sq19*, *sd12*, and *chl16,* respectively, grown in BG-11 medium supplemented with 40 mM NaCl. The specific growth rate and doubling per day were found to decrease with the increased NaCl concentration beyond the 40 mM (Tables 1, 2, and 3). Yao et al. [20] also reported similar results with *Tetraselmis subcordiformis*. Xia et al. [21] also observed the decreasing trend of biomass salinity-induced stress in *D. abundans* beyond particular salt concentration in cultivation media. In another study by Rao et al. [22] revealed the maximum biomass productivity with the *Botryococcus braunii* grown with the salinity-induced (34 mM of NaCl) media and a decreasing trend was observed beyond the 34 mM NaCl concentration. The patterns of stepwise cultivation strategies indicate the feasibility of using stepwise production strategies, which comprise a growth phase without any salinity stress and a lipid production phase with the induced salinity stress [21, 22].

Effect of Salinity on Lipid Production

Increase in NaCl concentration in the growth medium significantly enhanced the lipid content and lipid productivity of *Sq19*, *Sd12*, and *Chl16* isolates in both media. The highest lipid content of *Sq19, Sd12,* and *Chl16* isolates seemed to be equal to 18.28, 30.70, and 32.19%, respectively, using 160 mM of NaCl in BG-11 media. The salinity

Table 1 Effect of salinity on growth kinetics, lipid content, and lipid productivity of *Sq19* isolate

Media (mM)	Specific growth rate (μday^{-1})**	Doubling per day (K)*	Biomass (g l^{-1})***	Biomass productivity, Bp (g l^{-1} day^{-1})***	Lipids content, Lc (%)**	Lipid productivity, Lp (mg l^{-1} day^{-1})**
BG-11						
Control	0.089^{a}	0.128^{a}	0.568^{b}	0.038^{b}	14.663^{a}	5.570^{a}
40	0.129^{b}	0.185^{b}	0.742^{d}	0.049^{d}	15.717^{bc}	7.701^{bc}
80	0.104^{ab}	0.150^{ab}	0.721^{c}	0.048^{c}	13.407^{ab}	6.430^{ab}
160	0.108^{ab}	0.155^{ab}	0.711^{c}	0.047^{c}	18.280^{c}	8.590^{c}
320	0.089^{a}	0.128^{a}	0.549^{a}	0.036^{a}	13.580^{a}	4.880^{a}
CHU-10						
Control	0.124^{bc}	0.179^{bc}	0.486^{a}	0.032^{a}	6.841^{a}	2.189^{a}
40	0.130^{b}	0.187^{c}	0.621^{b}	0.041^{b}	5.629^{a}	2.195^{a}
80	0.106^{ab}	0.152^{ab}	0.593^{b}	0.039^{b}	5.615^{a}	2.189^{a}
160	0.097^{a}	0.139^{a}	0.591^{c}	0.039^{c}	7.055^{a}	2.892^{a}
320	0.113^{abc}	0.162^{abc}	0.490^{a}	0.033^{a}	6.128^{a}	2.022^{a}

*Significant at $\alpha = 5\%$, ** Significant at $\alpha = 1\%$, *** Significant at $\alpha = 0.1\%$, level of significance. a–g means in the column with same superscript letter are not significantly different ($\alpha = 0.05$) as measured by 2-sided Tukey's post hoc range test between isolates

Table 2 Effect of salinity on growth kinetics, lipid content, and lipid productivity of *Sd12* isolate

Media (mM)	Specific growth rate (μday^{-1})**	Doubling per day (K)*	Biomass (g l^{-1})	Biomass productivity, Bp (g l^{-1} day^{-1})	Lipids content, Lc (%)*	Lipid productivity, Lp (mg l^{-1} day^{-1})
BG-11 media						
Control	0.117 ^{ab}	0.169^{ab}	0.746^{a}	0.049^{a}	26.58^{b}	13.02^{a}
40	0.133^{b}	0.193^{b}	0.760^{a}	0.050^{a}	29.18^{b}	14.59^{a}
80	0.112^{a}	0.162^{ab}	0.583^{a}	0.038^{a}	28.46^{b}	10.81^{a}
160	0.098^{a}	0.141^{ab}	0.678^{a}	0.045^{a}	30.70^{b}	13.81^{a}
320	0.088^{a}	0.127^{a}	0.624^{a}	0.041^{a}	12.28^{a}	05.03^{a}
CHU-10 media						
Control	0.089^{a}	0.129^{a}	0.485^{a}	0.032^{ab}	17.16^{bc}	5.42^{b}
40	0.114^{a}	0.165^{a}	0.553^{a}	0.036^{c}	15.06^{b}	5.42^{b}
80	0.098^{a}	0.142^{a}	0.458^{a}	0.030^{a}	18.16^{c}	6.42^{b}
160	0.088^{a}	0.127^{a}	0.524^{a}	0.0349^{bc}	18.42^{c}	6.42^{b}
320	0.090^{a}	0.130^{a}	0.483^{a}	0.032^{a}	07.61^{a}	2.43^{a}

*Significant at $\alpha = 5\%$, ** Significant at $\alpha = 1\%$, *** Significant at $\alpha = 0.1\%$, level of significance. a–g means in the column with same superscript letter are not significantly different ($\alpha = 0.05$) as measured by 2-sided Tukey's post hoc range test between isolates

Table 3 Effect of salinity on growth kinetics, lipid content, and lipid productivity of *Chl16* isolate

Media (mM)	Specific growth rate (μday^{-1})*	Doubling per day (K)***	Biomass (g l^{-1})***	Biomass productivity, Bp (g l^{-1} day$^{-1)***}$	Lipids content, Lc (%)*	Lipid productivity, Lp (mg l^{-1} day^{-1})**
BG11 media						
Control	0.112c	0.162^{c}	0.487a	0.029^{a}	27.347^{a}	7.838^{a}
40	0.113^{c}	0.163^{c}	0.636^{d}	0.037^{d}	27.230^{a}	10.107^{b}
80	0.102^{c}	0.147^{c}	0.587^{c}	0.034^{c}	29.550^{a}	10.708^{b}
160	0.083^{b}	0.120^{b}	0.549^{b}	0.032^{b}	32.193^{b}	10.276^{b}
320	0.069^{a}	0.099^{a}	0.501^{a}	0.029^{a}	28.600^{a}	8.294^{a}
CHU-10 media						
Control	0.104^{b}	0.151^{b}	0.275^{c}	0.016^{b}	17.640^{b}	2.777^{b}
40	0.102^{b}	0.148^{b}	0.312^{e}	0.018^{d}	18.157^{b}	3.268^{b}
80	0.096^{b}	0.139^{b}	0.294^{d}	0.017^{c}	19.043^{b}	3.237^{b}
160	0.089^{ab}	0.129^{ab}	0.246^{b}	0.014^{a}	21.727^{b}	2.957^{b}
320	0.068^{a}	0.098^{a}	0.23^{a}	0.013^{a}	11.590^{a}	1.506^{a}

*Significant at $\alpha = 5\%$, ** Significant at $\alpha = 1\%$, *** Significant at $\alpha = 0.1\%$, level of significance. a–g means in the column with same superscript letter are not significantly different ($\alpha = 0.05$) as measured by 2-sided Tukey's post hoc range test between isolates

stress induced with the 160 mM NaCl in BG-11 media also brought lipid productivity of 8.59, 13.81, and 10.27 mg l^{-1} day^{-1} with the *Sq19, Sd12,* and *Chl16* isolates, respectively, which were seen to be more than controls (with 0% NaCl in BG-11 media). Similar trends of results were observed with the microalgal species grown in CHU-10 media with 160 mM NaCl concentration (Tables 1, 2, and 3). Enhanced lipid production on salinity supplementation has been also reported with *Dunaliella* sp. [6], *Scenedesmus* sp. CCNM 1077 [9], *Botryococcus braunii* [22], *Botryococcus braunii* (IPPAS H-252) [23], *Navicula* sp [24], and *Thalassiosira* [25]. The results of the present study also revealed the primitive role of salinity stress in triggering the lipid production in microalgae species. Microalgae accumulate lipids according to the growth conditions and environmental stress. The post hoc test (Tukey method) showed a significant difference in lipid content and the productivity of microalgal species isolates.

Effect of Stepwise Salinity Stress

Microalgae isolate *sd12* grown in BG-11 medium was supplemented with 160 mM NaCl after 18 days of cultivation. The lipid content was found to be higher (39.42%) with the

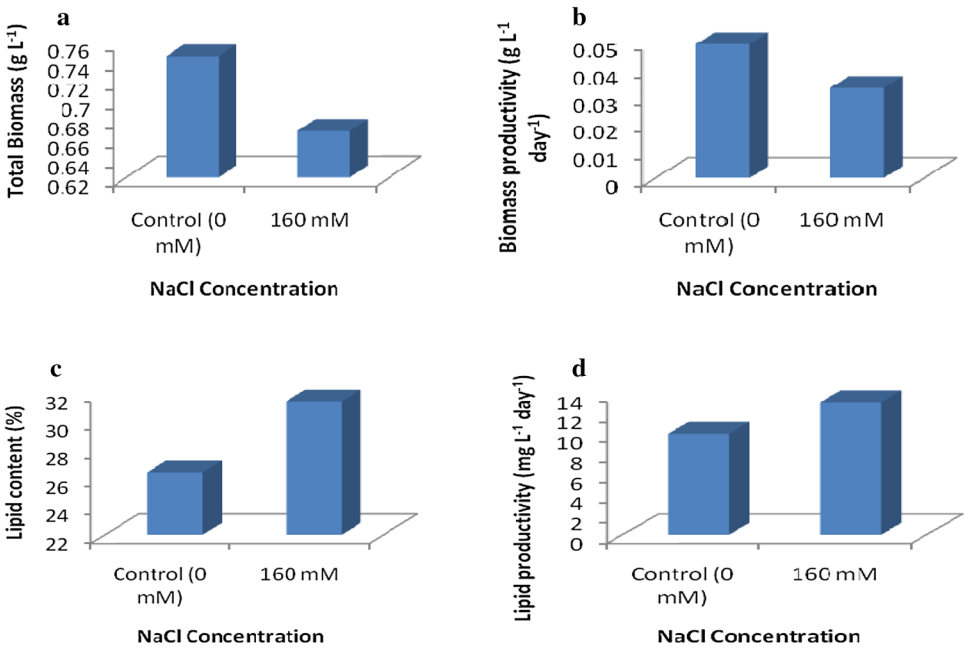

Fig. 3 Effect of two stage salinity on **a** biomass, **b** biomass productivity, **c** lipid content, and **d** lipid productivity of *Sd12* isolate

160 mM salinity stress (adopted after the growth phase) compared to the control (no salinity stress, 20.4%) (Fig. 3c). The lipid productivity has seemed to slightly improve with the execution of the stepwise cultivation strategy (Fig. 3d). The results indicate the feasibility of the stepwise cultivation strategy towards the enhanced lipid production. The improved lipid production has also reported in the case of *Dunaliella tertiolecta* by adopting the sodium azide intervention coupled with stepwise salinity stress cultivation [26]. Nagappana et al. [27] showcased the positive attributes of stepwise cultivation, which includes reduced bacterial contamination, targeted co-product generation, and enhanced biodiesel quality with the produced lipids. However, the microalgae need to be acclimatized well with the adopted stepwise salinity stress towards attaining the likely results.

Interaction Effects of Salinity Concentration, Media, and Microalgal Isolate on Algal Growth Kinetics and Lipid Productivity

The interaction effects of salinity concentration, microalgae isolate, and media on growth kinetics and lipid productivity have been analyzed through the general linear model method (three-way analysis of variance with contrasts). The concerned results have been presented in S1 Table (a–e). The specific growth rate (μ), shown a R^2 value of $>80\%$ with a P value of $\cong 0$, confirms the significance of the model. It can be seen that there are highly significant individual effect isolates and salinity concentration ($P \cong 0$) that of media. The interaction effect of all the combinations of factors on specific growth rate was substantial to expect for isolates*media. The results showcased the profound impact of salinity with different isolates and media. The significance of the model has further drawn through the R^2 values of biomass, and biomass productivity was found to be 0.873 and 0.963, respectively, with a p value of zero. In the case of biomass, the effect of isolates, interaction variables of media* salinity, and isolate*media*salinity were found to be non-significant. All the individual factors and their interaction have a significant impact on biomass productivity. Hence, the results showcased the salinity is having a profound effect on different isolates in the presence of various media. The R^2 value was significant with the lipid content ($R^2 = 0.976$, $P \cong 0.0$) and non-significant with lipid productivity ($R^2 = 0.415$, $P \cong 0.142$). The interaction effect of isolates*media and isolates*media*salinity was non-significant on total lipid content.

Conclusion

The microalgae isolates *Sq19*, *Sd12*, and *Chl16* performed well in BG 11 media supplemented with 40 mM NaCl, while lipid accumulation and productivity were found to be highest at 160 mM saline stress. The stepwise adoption of salinity stress (160 mM) with the best performed *sd12* microalgal isolate results in a slight improvement of lipid productivity. Further enhanced lipid productivity is feasible with the proper acclimatization of the algal isolate with the adopted stepwise salinity-induced cultivation strategy.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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