



## Effects of induced abiotic stresses on growth and lipid accumulation of the green alga *Stigeoclonium*

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### ABSTRACT

The fresh water microalga, *Stigeoclonium* sp., was examined for its eligibility as a biodiesel feedstock. The lipid content and growth performances were studied under normal culture conditions and abiotic stress conditions of nutrient deficiency, hypersalinity and pH stress using Chu-10 medium. Total lipid content was found to be 12.58% dry weight under nutrient replete condition which was enhanced by about 14% dry weight under nitrogen deficiency and by about 6% dry weight under phosphorus deficiency. Growth performances observed under different pH and salinity regimes concluded the ability of the organism to survive in varying environmental conditions. The observations suggest the suitability of *Stigeoclonium* as a viable source of biodiesel production.

**Key words:** Abiotic stresses; biodiesel; *Stigeoclonium*; total lipid content.

### INTRODUCTION

Currently, there is an enormous drive in the search for an alternative source of fuel other than the fossil fuels due to diminishing oil reserves and the ever-rising hike in fuel prices. The excessive use of these fossil fuels at the global level is creating an environmental hazard as they contribute to air pollution and global warming. The environmentally friendly and sustainable

biofuels could be a viable alternative to replace conventional petroleum.<sup>1</sup> Biofuels include biodiesel from microalgae and other oil crops such as soybean, sunflower, palm, peanut, animal fat, waste cooking oil, bioethanol and other alcohols from sugarcane and corn starch, H<sub>2</sub>, long-chain hydrocarbons and biogas.<sup>2,3</sup> However, animal and vegetable oils for biodiesel have proved to be highly expensive in terms of feed cost, thus rendering it unsuitable for large-scale commercialization. This has made microalgae a favorable source of cheaper biodiesel as it can be easily cultivated using air, water and sunlight as the basic requirements.

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Several microalgae species have high lipid content.<sup>4,6</sup> Various abiotic stress conditions like nutrient deficiencies, salinity stress, pH stress, temperature stress, light stress etc. have been found to increase the lipid accumulation in microalgae.<sup>7,8,9</sup> The biomass productivity, lipid cell content, and overall lipid productivity are some of the key parameters affecting the economic feasibility of algal oil for biodiesel production.<sup>10</sup> Several unicellular microalgae have been proposed as potential biodiesel feedstocks including *Botryococcus braunii*,<sup>8</sup> *Nannochloropsis* sp.,<sup>11</sup> *Chlorocella saccharophila*,<sup>12</sup> and *Haematococcus pluvialis*.<sup>13</sup> However, there are few reports on the filamentous microalgae for biodiesel production. They are superior to the unicellular species in the fact that they can be harvested easily with lower cost than unicellular algae. This study focuses on the green filamentous alga *Stigeoclonium* on its suitability for biodiesel production. The biomass production and lipid accumulations under different induced stress conditions were investigated.

## MATERIALS AND METHODS

Filaments of *Stigeoclonium* were collected from a small pond located at Bawngkawn, Aizawl, India. The filaments were homogenized and axenically cultured in Chu-10 medium containing KNO<sub>3</sub> (0.04g/l), K<sub>2</sub>HPO<sub>4</sub> (0.01g/l), CaCl<sub>2</sub> (0.04g/l), MgSO<sub>4</sub>·7H<sub>2</sub>O (0.025g/l), Na<sub>2</sub>SiO<sub>3</sub> (0.02g/l), Ferric citrate (0.003g/l), citric acid (0.003g/l), MnCl<sub>2</sub>·4H<sub>2</sub>O (0.5mg/l), Na<sub>2</sub>MoO<sub>4</sub>·2H<sub>2</sub>O (0.01 mg/l), H<sub>3</sub>BO<sub>3</sub> (0.5 mg/l), CuSO<sub>4</sub>·5H<sub>2</sub>O (0.02mg/l), CoCl<sub>2</sub> (0.04mg/l) and ZnSO<sub>4</sub>·7H<sub>2</sub>O (0.05mg/l) in 250 ml flasks. Cultures were maintained at 25°C under 10h:14h light-dark periods. The initial pH of the medium was adjusted to 7.5 using NaOH/HCl. Culture flasks are manually agitated at least twice daily to ensure proper nutrient mixing and light penetration.

Culture growth was estimated on the basis of optical density (OD) readings and total chlorophyll content. For monitoring the growth pattern, the culture flasks were homogenized by thorough shaking and 5 ml of the culture was

taken and OD at wavelength of 428 nm was recorded using UV-Vis spectrophotometer 117 (Systronics) for a period of 17 days at regular intervals of 2-3 days. Specific growth rate was calculated using the formula:  $\mu = \ln(n_2 - n_1) / \Delta t$ , where  $n_2$  and  $n_1$  are absorbance readings at which exponential growth is seen, and  $\Delta t$  = time interval of  $n_1$  and  $n_2$  in days.<sup>14</sup> For total chlorophyll estimation, fresh algal cells from 5 ml of culture were centrifuged at 12,000 rpm for 15 minutes. To the pellet, 5 ml of 80% acetone was added and left for 12 hours at 4°C. This was again centrifuged and OD of the supernatant was taken at 645 and 663 nm for quantifying total chlorophyll content by using the equation  $\text{Chl}(\text{mg/l}) = 8.02 \times \text{OD}_{663} + 20.21 \times \text{OD}_{645}$ .<sup>4</sup>

The effect of nutrient deficiency on growth and lipid accumulation was studied with modified medium composition as follows: (i) complete elimination of the phosphorus source K<sub>2</sub>HPO<sub>4</sub> (-P medium) (ii) complete elimination of the nitrogen source KNO<sub>3</sub> (-N medium) and (iii) control (full strength Chu-10 medium). The starvation conditions were applied for 7 consecutive days for lipid analysis and 17 days for observing growth performances and total chlorophyll content.

To study the effect of external pH on lipid accumulation, cultures were subjected to different initial pH values of 6.5, 7.5, 8.5 and 9.5 adjusted by using HCl or NaOH, and without using any buffer to avoid effect on culture and process of lipid synthesis in algae. Cultures were observed for 2 days for total lipid content and 17 days for observing growth performances.

The effect of different salinity levels (10, 20, 30, 40, 50 and 80 mM NaCl) on the growth and lipid content of *Stigeoclonium* was investigated. The alga was incubated for a period of 4 days for total lipid content and 7 days for observing growth performances.

For determining the total lipid content, a modified Bligh and Dyer's method was used.<sup>15</sup> Filaments were harvested by filtration and air-dried under sunlight for 3 days, and were grounded to fine powder and weighed. 1 gm of dried algal biomass was mixed with 30 ml of

chloroform: methanol (1:2) and sonicated using an ultrasonicator (Labsonic, Sartorius) for 2 min at full power. The mixture was vortexed for 20 min and allowed to settle for next 30 min. 15 ml of the supernatant was removed in another tube and 10 ml of chloroform was added to the mixture. This was vortexed for 10 min and 10 ml of water was added to it. The mixture was again vortexed for 1 min and then centrifuged at 2600 rpm for 10 min. The middle layer containing cell debris was discarded along with the upper methanol/water phase. The bottom chloroform/oil phase was collected and filtered into a pre-weighed test tube. The chloroform was completely evaporated at 40°C and the residual lipid content determined by weighing. Total lipid content was expressed as a percentage of the dry weight (DW) of the algae.

#### Statistical analysis

Statistical analyses of the results were performed by Student's t-test and treatments were compared with control.

## RESULTS AND DISCUSSION

### Effects of nitrogen and phosphorus deficiency on growth and chlorophyll content

The growth patterns of *Stigeoclonium* in the absence of nitrogen (-N) and phosphorus (-P), compared with the control (+N+P) for a period of 17 days are shown in Figure 1A. There is an initial lag phase up to 3 days after which the exponential phase commenced and continued till the 17<sup>th</sup> day when the experiment was terminated. P-deficiency showed an initial higher growth than the control but subsequently resulted in lower accumulation of algal biomass at the end of the experiment. This suggested that the algal growth was not inhibited by the absence of external phosphorus in the medium till up to more than 10 days after inoculation. Similar to this result, Chen *et al.*<sup>16</sup> also reported that phosphate deficiency in the medium did not inhibit the growth of the green alga *Dunaliella tertiolecta*. The absence of nitrogen however, had a marked impact on biomass accumulation as seen in the figure. This reduction in biomass

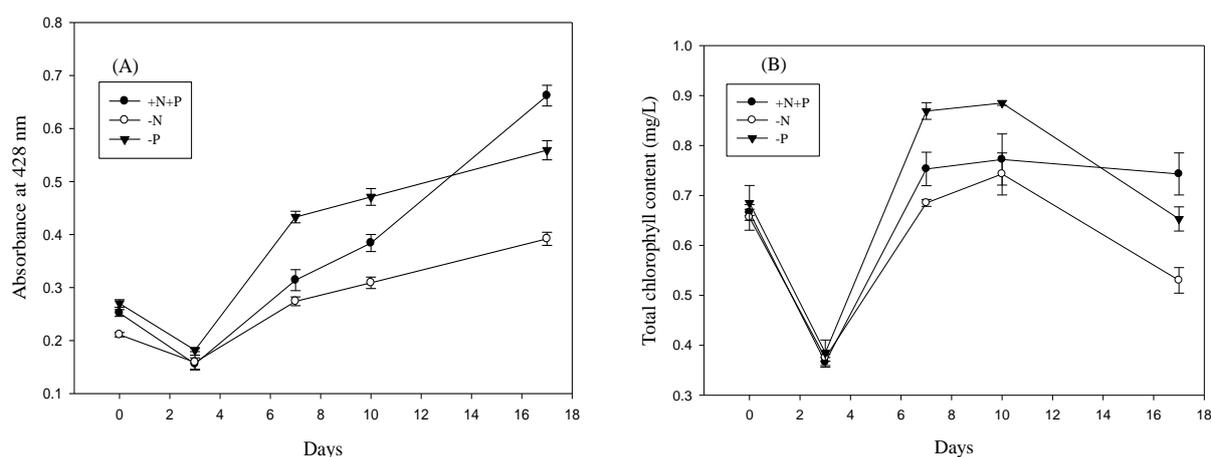


Figure 1. (A) The growth patterns of *Stigeoclonium* under control conditions (+N+P), nitrogen-free (-N) and phosphorus-free (-P) medium for a period of 17 days; (B). Total chlorophyll contents (mg/l) of *Stigeoclonium* under control conditions (+N+P), nitrogen-free (-N) and phosphorus-free (-P) medium for a period of 20 days. Vertical bars denote standard error of the mean. All values are presented as the mean  $\pm$  SE of three replicates.

production has also been reported for other species.<sup>12,17</sup>

The cell chlorophyll contents showed a progressive increase along with the increase in culture growth until a point is reached where cell chlorophyll content started to decline (Figure 1B). For the control, maximum cell chlorophyll content was seen on the 10<sup>th</sup> day with 0.772 mg/l and the corresponding culture OD was 0.384. This dropped to 0.743 mg/l on the 17<sup>th</sup> day with culture OD of 0.662. The total chlorophyll content dropped to  $0.530 \pm 0.0256$  mg/l under N-deficiency and under P-deficiency to  $0.653 \pm 0.0242$  mg/l after 17 days. The *Stigeoclonium* cultures under N-deficiency appeared relatively yellowish in colour due to decreased chlorophyll content in the cells as culture time increased. Li *et al.*<sup>10</sup> also reported a decreased pigment content for *Neochloris oleoabundans* under N-starvation. The specific growth rates for the three nutrient conditions are depicted in Figure 2. The specific growth rate of cultures grown under +N+P condition was the highest ( $0.24 \pm 0.001$  d<sup>-1</sup>). Lowest specific growth rate was seen under N-deficiency which showed about 1.7-fold decrease compared to control.

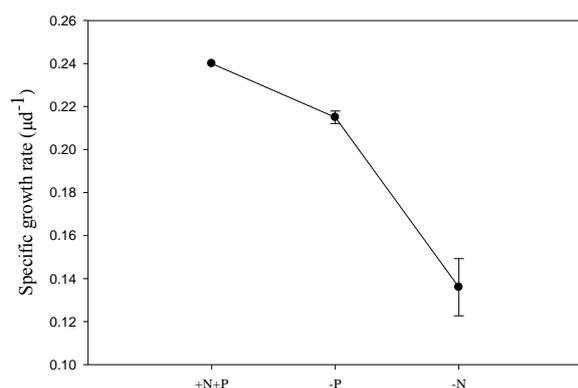


Figure 2. Specific growth rates of *Stigeoclonium* grown under control conditions (+N+P), phosphorus free (-P) medium and nitrogen free (-N) medium for a period of 17 days. All values are presented as the mean  $\pm$  SE of three replicates.

### Effect of medium pH on growth

Figure 3 depicts the growth pattern of *Stigeoclonium* under different pH regimes. The effect of initial medium pH on the specific growth rate of *Stigeoclonium* is shown in Table 1. Highest specific growth rate was seen at pH 6.5, a slightly acidic condition. At the higher pH levels tested, growth was seen to decrease with increasing alkalinity of the external medium. However, the decrease in the specific growth rate was not very pronounced. This is an added advantage for mass cultivation of this species as the results indicate that it can survive in varied pH conditions.

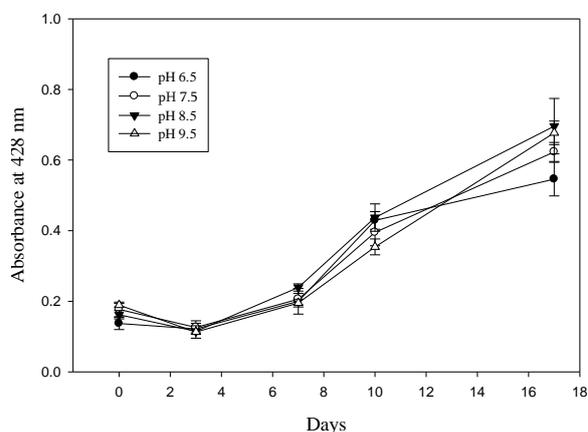


Figure 3. The growth patterns of *Stigeoclonium* under initial medium pH of 6.5, 7.5, 8.5 and 9.5 for a period of 17 days. Vertical bars denote standard error of the mean. All values are presented as the mean  $\pm$  SE of three replicates.

Table 1. Effect of different initial medium pH on the specific growth rate ( $\mu$ d<sup>-1</sup>) of *Stigeoclonium* after 17 days of treatment under light. Specific growth rate ( $\mu$ d<sup>-1</sup>) at pH 7.5 is taken as 100%. Values are presented as the mean  $\pm$  SE of three replicates.

Medium pH	Specific growth rate ( $\mu$ d <sup>-1</sup> ) Mean $\pm$ SE
6.5	$0.284 \pm 0.0081$
7.5	$0.240 \pm 0.001$
8.5	$0.22 \pm 0.005$
9.5	$0.218 \pm 0.005$

*Effect of salinity on growth*

*Stigeoclonium* was able to grow in all the tested concentrations of NaCl (10-80 mM) as shown in Figure 4. Maximum growth rate was observed in 20 mM salinity which resulted in a 35% increase in growth (Table 2). This was followed by slight reductions in growth with increasing NaCl concentrations but at 80 mM, the highest concentration tested, growth was still almost 100%. Therefore, it is concluded that this *Stigeoclonium* sp. exhibited a halophilic nature. Growth is favorable at salinity levels up to a certain level enabling it to tolerate saline environment without a pronounced effect on the lipid accumulation. Similarly, Rao *et al.*<sup>18</sup> also showed that the freshwater alga *Botryococcus braunii* could grow well in salinity levels of 17-85 mM NaCl.

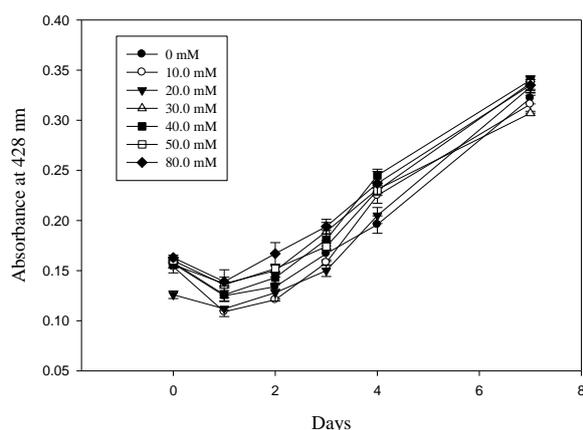


Figure 4. The growth patterns of *Stigeoclonium* at salinity levels of 0, 10, 20, 30, 40, 50 and 80 mM NaCl for a period of 7 days. Vertical bars denote standard error of the mean. All values are presented as the mean  $\pm$  SE of three replicates.

*Effects of N, P, pH and NaCl on lipid contents*

In *Stigeoclonium*, lipid accumulation was found to be enhanced by nitrogen and phosphorus deficiency. The lipid content was found to be 12.58% DW under control conditions (+N+P).

Table 2. Effect of different concentrations of NaCl on the specific growth rate ( $\mu\text{d}^{-1}$ ) of *Stigeoclonium* after 7 days of treatment under light. The NaCl was added to Chu-10 medium containing both N and P. Specific growth rate ( $\mu\text{d}^{-1}$ ) without NaCl in the medium is taken as 100%. Values are presented as the mean  $\pm$  SE of three replicates.

NaCl (mM)	Specific growth rate ( $\mu\text{d}^{-1}$ ) mean $\pm$ SE
0	0.240 $\pm$ 0.001
10	0.240 $\pm$ 0.001
20	0.324 $\pm$ 0.006
30	0.284 $\pm$ 0.002
40	0.256 $\pm$ 0.003
50	0.246 $\pm$ 0.001
80	0.237 $\pm$ 0.002

When cultures were subjected to N- and P-deficiency for a period of 7 days, the total lipids were found to increase to 26.20% DW under N-deficiency and 18.22% DW under P-deficiency (Figure 5A). According to Converti *et al.*<sup>19</sup> protein synthesis is inhibited by N-deficiency. At the same time, lipid synthesis continues at the expense of decreased protein synthesis when nitrogen is unavailable.<sup>20</sup> Therefore, microalgal cells have a high tendency to accumulate lipids under N-deficient conditions as observed for *Stigeoclonium* in the present study. Earlier to this, P-deficiency was also found to increase the total cellular lipid content in the alga *Monodus subterraneus*.<sup>21</sup>

The effect of initial medium pH on lipid yield was analyzed. It was found that lipid yield was not highly affected by initial pH at pH 6.5, 7.5 and 8.5 during this short incubation time. Although there were slight variations, there was no statistically significant difference among the three. At pH 9.5, the lipid content was reduced by about 5% (Figure 5B). This suggests that this *Stigeoclonium* strain will adapt well in a variety of pH without pronounced effect in the lipid accumulation. In contrast to this study, an earlier study reported that for *Scenedesmus* sp. and *Coccolastrella* sp., high medium pH resulted in higher lipid accumulation in the form of triacylglyc-

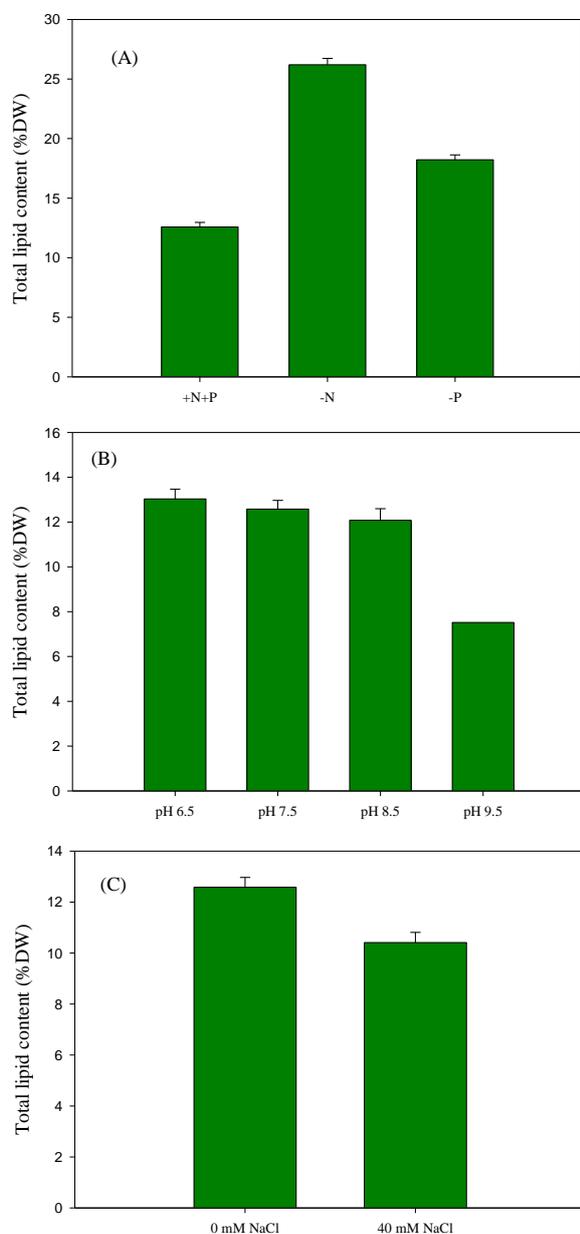


Figure 5. Effect of different abiotic culture conditions on the total lipid content of *Stigeoclonium* in terms of percentage dried biomass. (A) Control (+N+P), nitrogen deficiency (-N), and phosphorus deficiency (-P). Cultures were incubated for 7 days. (B) Different initial pH values of the medium. Cultures were incubated for 2 days. (C) Control (0 mM) and hypersalinity at 40 mM NaCl. Cultures were incubated for 4 days. All values are presented as the mean  $\pm$  SE of three replicates.

erols.<sup>22</sup>

Siaut *et al.*<sup>23</sup> reported a huge increase in cellular oil content of *Chlamydomonas reinhardtii* CC124 strain at 100 mM NaCl comparable to a level reached under nitrogen depletion. In the present study, when lipid content was tested at 40 mM NaCl, there was a 2.17% decrease under this salinity level when compared to the control (Figure 5C). Other studies have also found decreased lipid content in freshwater algal biomass at higher salinity.<sup>8</sup>

## CONCLUSIONS

Biodiesel of algal origin has been found to show similar performance compared to pure biodiesel in terms of fuel efficiency with a significant reduction in emission of hydrocarbons and nitrogen oxides.<sup>25</sup> The algal species are considered to be strong candidates for biodiesel production due to their higher photosynthetic efficiency, higher growth rates, high oil content and are easily cultivated. An important criterion for choosing algal species for biodiesel is to evaluate the cost of harvesting, since it is a significant capital and operating cost in any algal process.<sup>26</sup> Properties like large cell size, high specific gravity compared to the medium and reliable auto flocculation are involved in efficient harvesting.<sup>27</sup> Harvesting of unicellular microalgae involves centrifugation, sedimentation and flocculation, which heighten the production cost and time. In view of low-cost harvesting, dewatering and extraction of algal biomass,<sup>6</sup> the filamentous *Stigeoclonium* is recommended as it can be easily harvested using simple filtration techniques or using mesh screens. In this study, *Stigeoclonium* has been identified as a robust species which is relatively easy to culture using a simple nutrient medium. It can grow in a variety of pH and salinity conditions. It has been shown here that N- and P-limitation increased the overall lipid content, but further investigations are needed to see the combined effects with other stress conditions to increase lipid yield to a higher range in order to meet the requirements for biodiesel production. In addition to this, further research is re-

quired to enhance low-cost biomass production. In order to scale-up the biomass productivity, cultivation in an enclosed photobioreactor may be suggested,<sup>3</sup> with an additional input of CO<sub>2</sub> to increase lipids and triacylglycerols.<sup>28</sup>

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