The influence of temperature and nutrient concentrations on growth rate, biomass, Chlorophyll-\(a\), and biochemical compositions of \textit{Tetraselmis suecica} (Chlorophyta)

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Abstract. Microalgae have been got high attention due to its potentiality in aquaculture as live feed, and in industries as ingredients for pharmaceutical, cosmetics, and biofuel industries. The objective of this study was to know the optimum culture condition for profuse growth and biochemical composition of \textit{Tetraselmis suecica} under two parameters: temperature (20 °C, 25 °C, and 30 °C) and modified F/2 medium nutrients concentrations. Culture of group “A” 20°C was categorized as A1 (F/2 stock solution-A; 0.50 ml L\(^{-1}\) and F/2 stock solution-B; 0.20 ml L\(^{-1}\)), A2 (F/2 stock solution-A; 1.00 ml L\(^{-1}\) and F/2 stock solution-B; 0.40 ml L\(^{-1}\)) and A3 (F/2 stock solution-A; 1.50 ml L\(^{-1}\) and F/2 stock solution-B; 0.60 ml L\(^{-1}\)). Cultures in 25 °C and 30 °C were also categorized as groups “B” 25 °C (B1, B2, and B3) and “C” 30 °C (C1, C2, and C3), respectively. The culture was done for 2 weeks with L:D cycle of 12:12 by using fluorescent light. The highest biomass production was 0.80, 0.64, and 0.45 gL\(^{-1}\) in C2, B3, and A3, respectively. Biochemical analysis showed that protein; 21.92, 20.83, and 18.68 %, lipid; 10.76, 9.42, and 11.71 %, carbohydrate; 38.51, 37.78, and 41.49 %, ash; 15.89, 15.61, and 13.7 %, and moisture; 14.26, 15.02, and 14.42 % in biomass grown of “A”, “B” and “C” culture group, respectively. From the study, it could be said that \textit{T. suecica} is a eurythermal and mesotrophic habitant microalga which produce high protein and high carbohydrate in low and high temperature, respectively.

Keywords: Microalgae, media, culture condition, and biomass production

Track Name: Coastal Management and Marine Ecosystem
1. Introduction

Microalgae are microscopic photosynthetic organisms that are found in all aquatic environments. There are more than 40 species of microalgae with valuable properties that are used in aquaculture industries, and the algal biomass's lipid, carbohydrate, and protein composition determine its overall economic potential [1, 2]. Since they are a natural source of polyunsaturated fatty acids, they are a major food source for many aquatic organisms and the primary live feed component in marine hatchery operations [3]. In recent decades, Microalgae have been identified as a potential source of biofuel due to numerous advantages such as rapid growth potential, high biomass production rate, higher photosynthesis efficiency [4, 5], and can are grown in wastewater and on unproductive land [6]. Moreover, several chemical products and health foods have been mass-produced from microalgae, such as pigments, vitamins, long-chain unsaturated fatty acids, and antioxidants, for the nutraceutical industries [7].

Microalgae cultivation must be monitored and maintained to ensure optimal growth, high survival, biomass production, and enhancement of the biochemical composition due to their high metabolic flexibility in response to changes in environmental factors [8]. There is a continued need to establish factors that alter microalgal growth such as nutrients, light, temperature, salinity, and pH [9]. The growth rate of microalgae is influenced by the availability of nitrogen in the culture media, because it is a component of amino acid and chlorophyll [17]. Temperature is a fundamental environmental factor that strongly regulates microalgae growth. The temperature-growth range is ecologically important because it defines the temperature range in which microalgae can be metabolically active and determines their distribution [10]. Moreover, microalgal growth increases, and cell volume decrease with temperature [11]. There was no overall relationship between the percentage of microalgae biochemical compositions and temperature except ash (organic matter) [12], but it seems there is no consistent trend in the response of biochemical composition of microalgae to increases or decreases temperature [10].

_Tetraselmis sp._ are marine flagellate belong to Chlorophyceae, it grows as single, almost spherical, and motile cells with four equal flagella. They are used in relatively small amounts in aquaculture facilities as feed for shrimp larvae, rotifers, juvenile shellfish, and bivalve molluscs [13, 14]. This genus has also been discovered to have a wide range of antimicrobial activity [15]. In addition, its species have shown high potential as probiotics in fish [15]. Due of its high vitamin E content, _Tetraselmis sp._ have also been suggested as a source of vitamin E for human and animal consumption [16]. The temperature and nitrate concentration as the major causes of increasing cell abundance and growth rate of _Tetraselmis sp._ [17], _Tetraselmis suecica_ is a microalgae commonly used in aquaculture and is thought to be an optimal source of long-chain polyunsaturated fatty acids (PUFAs), especially eicosapentaenoic acid (EPA) [18], as well as the possibility of using it to remove N and P for purify wastewater [19]. Different culture conditions, such as temperature, salinity, nutrient, and light, may influence microalgae growth and proximate composition. Thus, this study was aimed to investigate the effect of temperature, within the range 20˚C, 25˚C, and 30˚C, and nutrient media concentrations on the growth and biochemical composition of _T. suecica_ that isolated from coastal water.

2. Materials and methods

2.1 Culture medium preparation and _Tetraselmis suecica_ isolation

2.1.1 Preparation modified F/2 medium (Guillard, 1975):

The modified F/2 culture medium stock solution was prepared as a concentrated solution, and for that natural seawater was filtered, thereafter, the different chemicals were diluted for making ‘A’ solution which was made with macronutrient and vitamins, and the solution ‘B’ was made with micronutrients most of them was trace metal that the microalgae need it in small amounts Table 1.
Table 1. Chemical composition of culture medium.

| A- solution | Amount | B- solution | Amount |
|-------------|--------|-------------|--------|
| Chemicals   | g.L⁻¹  | Chemicals   | g.L⁻¹  |
| NaNO₃       | 290.00 | FeCl₃·6H₂O  | 15.00  |
| KNO₃        | 50.00  | H₃BO₃      | 10.00  |
| Ca(NO₃)₂·4H₂O | 5.00  | Co(NO₃)₂·6H₂O | 2.00  |
| Mg(NO₃)₂    | 5.00   | K₂Cr₂O₇    | 0.50   |
| K₂HPO₄      | 65.00  | CuSO₄·5H₂O | 2.00   |
| KH₂PO₄      | 15.00  | MnSO₄·H₂O  | 1.00   |
| K₂SO₄       | 15.00  | ZnCl₂·6H₂O | 2.50   |
| MgSO₄·7H₂O  | 5.00   | Na₂-EDTA   | 5.50   |
| Urea        | 2.50   |             |        |
| HNO₃        | 125.00 ml.L⁻¹ |         |        |
| H₃PO₄       | 15.00 ml.L⁻¹ |          |        |

Vitamins

| Thiamin     | 10.000000 |
| D-Biotin    | 0.02500   |
| Cyanocobalamin | 0.00001  |

2.1.2 Isolation steps of Tetraselmis suecica

*Tetraselmis suecica* was isolated from coastal water and the isolation was done following the methods described by Affan et al [20]. In summary, a 1 mL diluted sample was transferred to a Sedgwick Rafter (S–R) counting chamber, then target species was isolated with a mouth-sucking micropipette (MSM) technique Affan et al [21]. To make MSM, the tip of a glass pasture pipette was heated over and drawn out, the MSM needle was positioned close to the *T. suecica* which was checked using an inverted microscope (MEIJI Techno- TC5100, JAPAN), and a gentle suction was applied to suck or picked up the single cell. Thereafter, *T. suecica* was transferred to a tissue culture multiwell plate for growing and subculture in autoclaved seawater which was enriched with F/2 medium nutrients. Then, the single species was again cultured in agar (Hi Media Laboratories, Ltd. India). To prepare agar culture medium, 2 % of agar [21] (w/v) and F/2 medium of ‘A’ and ‘B’ stock solution (0.5 mL of A- solution and 0.2 mL of B- solution) added and diluted, then autoclaved. *T. suecica* from the multiwall was transferred and grown in the agar plate. The agar plate was kept at a temperature of 25 °C under fluorescent light (180 µmol photon.m⁻² s⁻¹) on a 12:12 light:dark (L:D) cycle and the culture plate was checked every two days to observe the colony of *T. suecica*.

2.1.3 Determination of optimum growth conditions

For determination of the optimum growth condition the culture experiments were designed as follows; The culture was grown in three different temperatures (20 °C, 25 °C and 30 °C) with three concentration of F/2 medium with fixed light intensity (as used during isolation) and salinity and pH of 38.00 psu and 8.00 psu, respectively of natural seawater. Each 1 L flask with 250 mL culture media was inoculated with approximately 20 cells ml⁻¹ of *T. suecica*. The cultures were grown under fluorescent lights (180 µmol photons. m⁻² s⁻¹) on a 12:12 light: dark (L: D) cycle for 2 weeks.
Table 2. The culture conditions temperature and nutrients concentrations.

| Temperature | Solution “A” ml L⁻¹ | Solution “B” ml L⁻¹ |
|-------------|---------------------|---------------------|
| Group “A”   | A1: 0.5, A2: 1, A3: 1.5 | A1: 0.20, A2: 0.40, A3: 0.60 |
| Group “B”   | B1: 0.5, B2: 1, B3: 1.5 | B1: 0.20, B2: 0.40, B3: 0.60 |
| Group “C”   | C1: 0.5, C2: 1, C3: 1.5 | C1: 0.20, C2: 0.40, C3: 0.60 |

2.1.4 Estimation of dry biomass and cell abundance:
Each treatment was replicated twice. A 5 mL sample was collected from each culture flask on every two days and fixed with Lugol’s iodine solution. Then, a 1 mL fixed sample was used for counting using with a Sedgwick-Rafter (S-R) counting chamber under an inverted microscope at 400x magnification. A 30 mL sample was collected for biomass estimation from each culture on same time the sample was collected for cell counting. The sample was filtered through pre weighed GF/C Whatman filter paper. A pre-weighed filter paper that was soaked in distilled water and dried at the same time as a blank. The biomass content filter paper was kept at 55°C in an oven, dried and weighed, and the dry weight biomass was calculated as gL⁻¹, which was plotted as a growth curve. The specific growth rate (l), defined as the increase in cell density per unit time (Pirt 1975)[23], and formulated as follows:

\[
\mu_{\text{max}} \text{ (day}^{-1}\text{)} = \ln \left( \frac{X_{1}}{X_{0}} \right) / \left( t_{1} - t_{0} \right)
\]

Where \(X_{0}\) and \(X_{1}\) are the biomass at the beginning \((t_{0})\) and the end \((t_{1})\) of the selected time interval between inoculation and maximum biomass production.

2.2 Estimation of Tetraselmis suecica chlorophyll a:
Chlorophyll \(a\) of \(T. \) suecica was estimated following the method described by Parsons et al [23], the short description of that respective method; the preparation of chlorophyll- \(a\) samples from 30 mL of culture aliquots which was filtered by standard vacuum filtration through a 0.45 \(\mu\)m pore size whatman GF/C filter paper. The filter paper was chopped up to extract the pigments, then placed in 10 mL of 90 % acetone in a 25 mL universal tube and stored at 4°C for 24 hours to allow time for the \(T. \) suecica pigments. After extracted period, the samples was removed from the freeze and allowed to warm at room temperature in a dark place. The acetone volume was restored to 10 mL, the solution was then centrifuged (ALC Multi Speed Refrigerated Centrifuge PK 121 R) for 5-10 min at 3000-5000 rpm. Finally, the chlorophyll- \(a\) concentration was determined using a Spectrophotometer (UV-VIS - mini 1240.), and was calculated using the formula described by Parsons et al [24],

\[
\text{The chlorophyll } a \mu g L^{-1} = \frac{(27.6 \ast (A665 - A750)) \ast s}{v}
\]

\(A665=\) absorbance at 665 nm, \(A750=\) absorbance at 665 nm, \(s = \) solvent extract volume, ml, \(v = \) solvent extract volume, ml.

2.3 Biochemical Composition Analysis
The biomass from the best growth condition was selected for biochemical analysis, and the best growth condition was selected based on higher biomass production among the different culture conditions. The sample was harvested by centrifuged (ALC Multi speed, PK 121R, Korea) for 15 min at 4000 rpm Price et al [24] then preserved in -80°C temperature and finally freeze-dry powder for further study (Mini Lyotrap LTE, PE114732, Great Britain). The protein, lipid, carbohydrate, moisture and ash contents were
determined following the methods of the Association of Official Analysis Chemists AOAC (2005) [25], such as crude lipid was determined by Soxhlet extraction; crude protein, by the Kjeldahl method; ash, by calcinations in a furnace at 550° C (Jelrus P.D.Q-U.S.A.); and moisture by heating at 105° C for 20 h.

2.4 Statistical analysis
Statistical package for social sciences (SPSS 20.0) were applied for one way ANOVA test in the evaluation of the differences in the values followed by Tukey’s tests. A P-value of <0.05 was considered as significant.

3. Results
There is no doubt that Tetraselmis suecica is useful in aquaculture, in the bioconversion of solar energy, and potentially useful as food for human consumption [26]. Therefore T. suecica was isolated and studied its growth response under conditions of temperature and nutrients (Table 2.).

3.1 Growth characteristics biochemical composition for T. suecica

3.1.1 Dry biomass production
The results showed the dry biomass of T. suecica varied from 0.33 to 0.45 g L\(^{-1}\) in "A" group and was highest in A3, followed by A2 and A1 (Figure 1A). In the "B" group, the biomass production ranged from 0.51 to 0.64 gL\(^{-1}\), with the highest value in B3, followed by B1 and B2 (Figure 1B). In the "C" group; the biomass production was varied from 0.62 to 0.80 gL\(^{-1}\), and the highest was in C2, followed by C3 and C1 (Figure 1C). Maximum dry biomass was higher than that of all other culture conditions in C2.

Figures 1A, 1B, and 1C the dry biomass production of T. suecica at “A”, “B”, and “C” groups in F2 medium concentrations of A1, A2, A3, B1, B2, B3 and C1, C2, C3.
3.1.2 Specific growth rate and cell abundance

The *T. suecica* grew under all culture conditions (temperature and F2 media) with different cell densities (represented by the column chart). The maximum specific growth rate (μ_max d⁻¹, represented by the line chart) was estimated as an informative way to ascertain microalgae activity, which can increase at exponential rates. The growth rate of microalgae varies greatly depending on the temperature, and the findings of our investigation into this phenomenon are presented in table 3. The highest mean values of specific growth rate (μ_max d⁻¹) of *T. suecica* was observed in culture at 25°C (0.41 ± 0.007 day⁻¹). The cell densities were the greatest in the 30°C water temperature condition (Figure. 2C), followed by 25°C (figure. 2B) and 20°C (figure. 2A). The highest μ_max occurred at 10th day after inoculation in all treatments except the 20°C water temperature condition, where, the highest μ_max was 0.37d⁻¹ and occurred on day 12 after being inoculated with A2 nutrient concentration. The highest cell density 5.51×10⁵ cells mL⁻¹ occurred at the μ_max of 0.36 d⁻¹ on day 10th with A3 nutrient concentration in 20°C (Figure 2A). In "B" culture, the maximum cell density was found 5.38 ×10⁵ cells mL⁻¹ on day 10 with B2 nutrient concentrations conditions, while the highest μ_max was 0.42 d⁻¹ in B3 culture (Figure 2B). Similarly, in "C" culture, the highest cell density was 5.72 ×10⁵ cells mL⁻¹ with the highest μ_max 0.37 d⁻¹ on day 10th of culture (Figure 2C).

| Temperature groups (° C) | 20      | 20      | 30      |
|--------------------------|---------|---------|---------|
| *Tetraselmis suecica*    | 0.36 ±0.008 | 0.41 ±0.007 | 0.35 ± 0.03 |
3.1.3 Chlorophyll- a of Tetraselmis suecica

The chlorophyll-a concentration ranged from 0.23 to 3.10 mg L\(^{-1}\) and was the highest concentration in A1, followed by A3 in "A" culture group (Figure. 3A). In the "B" group, the chlorophyll a concentration varied from 0.25 to 3.41 mg L\(^{-1}\) with the highest in B2, followed by B3 (Fig. 3B).

Similarly, in the "C" group, the chlorophyll- a concentration ranged from 0.24 to 3.94 mg L\(^{-1}\), and
the highest was in C2, followed by C3 (Figure 3C). Among nine culture conditions, the highest chlorophyll-\(a\) was found in C2 culture. Statistically, the results showed that there was no a significant difference of cell abundance and chlorophyll- \(a\) between nutrients concentrations (\(P<0.05\)) for all temperature treatments.

Figure 3A, 3B, and 3C Chlorophyll \(a\) concentrations at “A”, “B”, and “C” groups in F2 medium concentrations of A1, A2, A3, B1, B2, B3 and C1, C2, C3 respectively.
3.1.4 Biochemical composition analysis

The biochemical composition of *T. suecica* determined as proteins, carbohydrates, total lipid, moisture, and ash in percentages of dried biomass of "A", "B", and "C" culture conditions. The *T. suecica* biochemical composition was as follows: protein; 21.92 %, 20.83 %, and 18.68 %, lipid; 10.76 %, 9.42 %, and 11.71%, carbohydrate; 38.51 %, 37.78 %, and 41.49%, ash; 15.89 %, 15.61 %, and 13.7%, and moisture; 14.26 %, 15.02 %, and 14.42 % in culture biomass grown in temperature of "A", "B", and "C", respectively (Figure 4).

![Biochemical composition analysis](image)

**Figure 4.** Biochemical compositions analysis at 20°C, 25°C and 30°C.

4. Discussion

4.1 Effect of temperature and nutrients on growth

*Tetraselmis sp.*, is one of the species isolated from coastal waters that is characteristic of mid-latitude areas with significant annual temperature fluctuations and has been widely used in aquaculture around the world [27]. In this study, isolation of a unialgal strain of *Tetraselmis suecica* (Chlorophyceae) from the direct seawater sample. The combination of MSM and agar culture techniques for the isolation of green algae was more effective and less time-consuming than serial dilution and agar plate culture isolation technique or direct pipetting. This species grew well in all culture conditions with remarkably highest cell abundance and chlorophyll-α concentration in C3 and C2 culture respectively. Therefore, it can be said that this species is euryhaline and mesotrophic and it makes bloom or vigorous growth at the temperature of 30°C. This result is in line with the previous study by Rukminasari et al [17], where their results indicate that the highest growth rate of *Tetraselmis sp.* was found at 30°C with double nitrate of Conway medium. The microalgal growth rate is expected to increase with temperature within range, and then rapidly decline above a critical temperature [28]. In this study, *T. suecica* reached the highest peak of growth at the temperature of 30°C with nutrients concentrations of C culture on day 10 of cultivation. This finding supported by previous study by Kim et al [44] who found that the highest cell concentration of *Tetraselmis sp.* showed after 10 days. *T. suecica* produced the highest chlorophyll-α value of 3.94 mg L⁻¹ at C2. Previous research conducted by Ginting et al [29] measured the values of chlorophyll-α of *Tetraselmis sp.* was 48 μg mL⁻¹ and 40.88 μg mL⁻¹, and Sani et al [30] found total chlorophyll between 3.65-19.20 mgg⁻¹ from dried biomass. The decrease in chlorophyll α content of *T. suecica* was most pronounced in the nutrient-stressed cultures [31], these
results were in agreement with our findings of chlorophyll-α content. In addition, these results explain there is an ideal temperature range in which a specific microalgal strain shows the best growth performance [32].

4.2 Effect temperature and nutrients on biochemical compositions

The temperature plays an important role in controlling the physiological and biochemical processes in algae and their response to temperature changes varied with species [10]. High growth temperature has been linked to a substantial reduction in protein content, as well as increases in lipid and carbohydrate content [33, 34]. In our results, the lipid contents were low, due to the presence of significant amounts of ash [37]. There were no significant differences in the average crude protein content between different temperatures. The biochemical composition of microalgae often responds strongly to nutrient stress [35]. Under some conditions, total lipid content varies between microalgal species, ranging from very low 1 % to 75 % but it can exceed to very high 90 % of dry weight [36]. The biochemical composition of microalgal species (even in the same culture medium) can vary across culture conditions. A previous study reported the protein content was 70.24 %, 68.01 %, 68.67 %, 64.58 % and 62.81 % while the carbohydrate content was 9.88 %, 11.68 %, 12.72 %, 15.57 % and 19.63 % when *Spirulina maxima* was cultured in the same medium at 20° C, 25° C, 30° C, 35° C and 40° C [38]. Our result also showed low protein and high carbohydrate content at high temperature. Lipid content did not show any trend to increase or decrease with nutrient concentration and temperature rises and falls. Zheng et al [39] reported that plants adapt to temperature change is to decrease the degree of unsaturation of membrane lipids under high temperature and increase it under low temperature. Oliveira et al. [34] also found lipid content of 6.22 % and 6.22 % in *Spirulina maxima*, and 7.24 % and 6.32 % in *Spirulina platensis* when those species were culture at temperature of 20° C and 25° C. The lipid content of *T. suecica* was low at high temperatures which are similar with previously mentioned studied, and according to the results obtained the carbohydrate was high under all conditions of temperature, the cause is this species is one of the microalgae that has been found to accumulate large quantities of carbohydrates [37]. The lipid contents were low, due to the presence of significant amounts of fibers and ash. However, the lipid content of *T. suecica* in this study showed a minor increase with increasing temperature. This was in agreement with the previous report that the lipid content of *T. subcordiformis* increased with increasing temperature [40]. The ash content of *T. suecica* in this study was higher at 20° C being similar to the values obtained for the same species at the same temperature [39] and in other species such as *Tetraselmis sp.* and *T. chuii*. [37].

Several of the different descriptions of temperature effects on the growth and biochemical compositions of *Tetraselmis spp.* have been reported. At 20° C *T. subcordiformis* had the highest lipid content (22.25 %) [38], which corresponded to its optimal growth temperature. On the other hand, temperatures for optimal growth rates and highest biomass production of *T. chuii* were ranged from 20° C to 30° C [42]. Our study registered the carbohydrate contents were the highest value followed by protein and lipid these results agree with a previous similar study carried out on *T. suecica* cultured under 21° C and F/2 medium [43].

5. Conclusion

It's crucial to know how microalgae respond to growth and adapt to temperature changes and nutrients concentration. Based on the results of the study, it was concluded that the effect of temperature and nutrients on optimal growth and biochemical content of *Tetraselmis sp.* depends on the varied species, and could be increasing or decreasing by manipulating the culture conditions (temperature and media concentrations). *Tetraselmis suecica* showed maximum growth rates, cell density, biomass, and chlorophyll-α in higher temperatures 30° C and a 1, 1.5 ml L⁻¹ of solution A and 0.40 ml L⁻¹, 0.60 ml L⁻¹ of solution nutrients concentration of F2 media. An increase in the carbohydrate and lipid contents has been observed as temperatures rise, while the inverse was observed with protein content.
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