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Enhancement Of Green Algal Growth Using *Hypnea Musciformis* And *Zonaria Variegata* Seaweed Liquid Fertilizer

[1]Divyalakshmi.R, [2]Lakshmi.D, [3]Roshini. S

[1]Research Scholar, [2]Associate Professor, [3]PG Student
Department of Plant Biology and Biotechnology,
Shrimathi Devkunvar Nanalal Bhatt Vaishnav College for Women,
Chromepet, Chennai, 600 044, Tamil Nadu, India

ABSTRACT:

Microalgae can be used as a renewable energy source, as nutritional supplements, and as a source of pharmacological compounds. However, playing in insufficient light conditions usually limits their optimal growth. The research examines the efficacy of red and brown macroalgae-based Seaweed Liquid Fertilizers (SLFs) derived from *Hypnea musciformis* and *Zonaria variegata* in stimulating the development of green microalgae. Microalgal cultures were treated with the SLFs to evaluate their effects on growth rate, biomass yield, photosynthetic efficiency, and biochemical composition. The findings indicated that when macroalgae-derived SLF was used, algae grew and exhibited higher biochemical productivity than in the control cultures. The findings demonstrate the opportunities of macroalgae-based SLF as a natural growth factor for the extensive growth of microalgae, with sustainable potential for biofuel production, bioproduct synthesis, and environmentally friendly aquaculture development.

Keywords: Macroalgae, Seaweed Liquid Fertilizer (SLF), Green algae, Antibacterial, Antioxidant.

I. INTRODUCTION

Marine macroalgae, also known as seaweeds, play a significant role in nutrient cycling, carbon capture, and ecological stability in aquatic environments [1]. They contain bioactive compounds, such as phytohormones, amino acids, vitamins, minerals, and sulfated polysaccharides, which are natural biostimulants and biofertilizers and can thus increase nutrient uptake, photosynthetic activity, stress tolerance, and overall growth in plants and microalgae [2, 3]. Seaweed formulations are eco-friendly alternatives to chemical fertilizers, with Seaweed Liquid Fertilizers (SLFs) being particularly productive and environmentally sustainable [4, 5].

Microalgae are photosynthetic eukaryotes, multicellular, capable of rapid biomass growth and production of useful biomolecules such as proteins, lipids, pigments, and antioxidants, and therefore have the potential to be discovered as useful biotechnology, biofuel, and nutraceutical candidates [6, 7]. However, large-scale microalgae cultivation is often constrained by nutrient limitations and the cost of purchasing synthetic media [8]. Macroalgae-derived SLFs offer a sustainable solution by supplying nutrients and

natural growth regulators that stimulate algal metabolism, pigment accumulation, and biomass production [5, 9, 10].

The red and brown macroalgae differ in their biochemical composition and bioactivity possibilities. Carrageenans, phenolics, and antioxidants are present in the red alga *Hypnea musciformis*, which enhances their growth and protects cells from oxidative stress [11, 12]. Conversely, the brown alga *Zonaria variegata* contains high levels of phlorotannins, alginates and fucoxanthin, which have high bioactive and protective properties [13, 14]. Although they have potential, there is a lack of comparative studies determining the impact of red and brown algae SLFs on green microalgae.

Thus, the current work assesses the impact of *Hypnea musciformis* and *Zonaria variegata* SLFs on growth, chlorophyll a, chlorophyll b and β-carotene levels; biochemical composition (protein and carbohydrates) and bioactivities (antibacterial and antioxidant activities, SOD, POD, catalase, proline, DPPH and $ABTS^{\cdot+}$). The results will contribute to the development of sustainable biofertilisation techniques, ensuring improved algae growth [5,15].

II. MATERIALS AND METHODS

2.1 Microalgae Cultures and Maintenance

Chlorella sp. and *Desmodesmus sp.* were collected from the culture collection of SDNB, Vaishnav College for Women, Chromepet, Chennai-44 (Fig. 1). The cultures were kept in Bold's Basal Medium (BBM) at 24 ± 1°C in a thermostatically controlled room under a 12:12 h light/dark daily photoperiod provided by fluorescent tubes. Cultures were sub-cultured every 7 days to sustain exponential growth.

Fig 1: Microalgae (a) *Chlorella* sp. (b) *Desmodesmus* sp.

2.2 Collection and Preparation of Seaweed Samples

Hypnea musciformis (Red) and *Zonaria variegata* (Brown) macroalgae were collected from R.K. Algae Project Center, Ramanathapuram, India. (Fig 2) Samples were cleaned in tap and sterile distilled water to remove debris and epiphytes, dried for 4-5 days, and ground to a fine powder. Powdered macroalgae prepared SLF at concentrations of 0.5%, 1% and 2% (v/v) in BBM. The prepared SLF-amended media were used to grow *Chlorella sp.* and *Desmodesmus sp*. under the same laboratory and maintenance conditions.

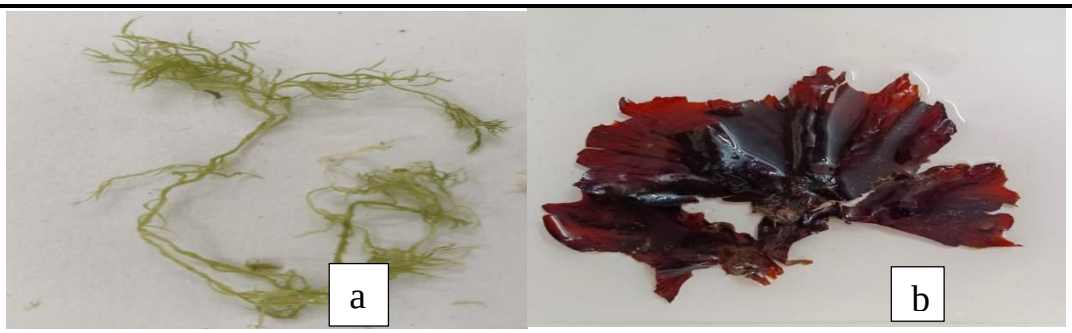


Fig 2: Fresh specimens of *Hypnea musiformis* and *Zonaria variegata.*

2.3 Growth Study

The growth of the algae was observed over 14 days. The samples (5 mL) were collected every other day and centrifuged at 5000 rpm for 10 minutes, followed by analysis of pigments (Chlorophyll-a, Chlorophyll-b, β-carotene), proteins, and carbohydrates [16].

2.4 Pigment Estimation

Chlorophyll a, Chlorophyll b and β-carotene were extracted in 80% of acetone and spectrophotometrically measured at 663 nm for chlorophyll a, 645 nm for chlorophyll b and 450 nm for β-carotene, respectively [17,2 and18].

2.5 Protein Estimation

The total protein was sonicated in phosphate buffer (0.1 M, pH 6.8), extracted and the Coomassie Brilliant Blue G-250 was measured at 595 nm with BSA as a reference point [19].

2.6 Carbohydrate Assessment

The total carbohydrates were determined using the phenol-sulfuric acid method using D-glucose as a standard [20].

2.7 Phytochemical Screening

Standard chemical tests were used to qualitatively analyze secondary metabolites (alkaloids, saponins, tannins, flavonoids, cardiac glycosides, quinines, coumarins, phenols, terpenoids and phlobatannins) in algal extracts. Presence or absence was determined either by the color change or precipitate formation [21,22].

2.8 Antibacterial Assay

Bacterial strains (*Escherichia coli, Bacillus subtilis, Klebsiella pneumoniae, Staphylococcus aureus, Pseudomonas aeruginosa*) were taken into the SDNB culture collection of Vaishnav College. Ethanol, methanol, ethyl acetate, acetone, and DMSO (5 g seaweed in 20 mL solvent, 20 days) were used to prepare seaweed extracts, which were filtered and placed on a sterile disk. Antibacterial activity was assessed by the diffusion technique on Mueller-Hinton agar plates, with positive controls consisting of standard antibiotic discs and negative controls consisting of solvent-only discs [23,24]. Zones of inhibition have been measured in mm.

2.9 Antioxidant Assays

2.9.1 Superoxide Dismutase (SOD) Activity

SOD activity was measured by the inhibition of the photochemical reduction of nitroblue tetrazolium (NBT). The 100 mL algal samples were homogenized with 5 mL of 50 mM phosphate buffer (pH 7.8), and the mixture was centrifuged at 12,000 rpm (3 milliliters). The reaction mixture (3 ml) contained 2.8 ml of phosphate buffer, 0.1 ml of methionine, 0.1 ml of NBT, 0.1 ml of riboflavin, and 0.1 ml of enzyme extract. Tubes were exposed for 10 minutes, and their absorbance at 560 nm was measured [25]. To measure SOD activity, the percentage of NBT reduction was used.

2.9.2 Peroxidase (POD) Activity

POD activity was measured as the rate of hydrogen peroxide (H_2O_2) consumption, used to monitor guaiacol oxidation. To homogenize and centrifuge 100 milliliters of algal samples at 12,000 rpm for 15 minutes in a 0.1M Tris-HCl buffer at pH 7.4, the following solution was added [26]. The reaction mixture contained phosphate buffer (2.8 mL), guaiacol (0.1 mL), H_2O_2 (0.1 mL), and enzyme extract (0.1 mL) (3 mL). The absorbance (470nm) increased after 3 minutes and was determined using an extinction coefficient of 26.6 $mM^{-1}cm^{-1}$.

2.9.3 Catalase (CAT) Activity

The CAT action estimated the breakdown of H_2O_2. One hundred milliliters of algal samples were homogenized using 1 mL of 0.1M Tris-HCl buffer (pH 7.4) and centrifuged at 12,000 rpm after 15 minutes. The reaction comprised 2.9 mL phosphate buffer, 0.1 mL H_2O_2, and 0.1 mL enzyme extract [25]. The reduction in absorbance at 240 nm was monitored for up to 3 min, and catalase activity was calculated using an extinction coefficient of 39.4 $mM^{-1}cm^{-1}$.

2.9.4 Proline Estimation

The acid-ninhydrin technique was used to determine the proline content. Scientific samples (100 mL) were homogenized with 5 mL 3% sulfosalicylic acid and centrifuged at 10,000 rpm for 10 minutes. To produce a 2 mL extract, 2 mL of acid-ninhydrin and 2 mL of glacial acetic acid were added to the 2 mL of extract, which was incubated at 95 °C for 30 minutes before cooling and extraction with 4 mL toluene. The toluene layer was measured by absorbance at 520 nm, and the proline content was measured using a standard curve of L-proline [27].

2.9.5 DPPH Free Radical Scavenging Assay (FRSA)

DPPH radical scavenging activity was determined with the help of the 1,1-diphenyl-2-picrylhydrazyl. The algal extracts were combined with the DPPH solution and incubated in the dark for 30 minutes. The absorbance at 517 nm decreased, and the percentage inhibition was calculated [28].

2.9.6 $ABTS^{\bullet+}$ Radical Cation Scavenging Assay

ABTS+ radical cation was used to perform the ABTS test. The algal extracts were added to the ABTS+ solution and incubated for 10 min. The absorbance was read at 734 nm. Radical scavenging activity was expressed as the percentage of inhibition [29].

III. RESULT AND DISCUSSION

3.1 Phytochemical Composition

Hypnea musciformis and *Zonaria variegata* displayed a high concentration of both alkaloids, saponins, tannins, flavonoids, terpenoids, and cardiac glycosides **(Table 1)**, whereas there was no phenol or

coumarin present [21]. These bioactive substances promote the physiological functions of plants and microalgae, thereby enhancing nutrient absorption and metabolic activities.

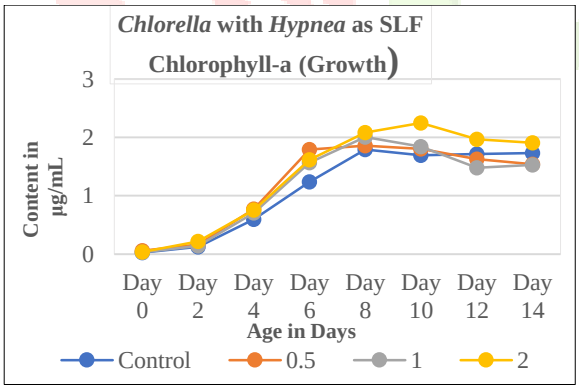
S.No	Phytochemicals	*Hypnea musciformis*	*Zonaria variegata*
1	Alkaloids	+	+
2	Saponins	+	+
3	Tannins	+	+
4	Flavonoids	+	+
5	Cardiac Glycosides	+	+
6	Quinines	+	+
7	Caumarines	-	-
8	Phenols	-	-
9	Terpenoids	+	+
10	Phlobatannins	+	+

Table 1: Phytochemical constituents of the two macroalgal samples.

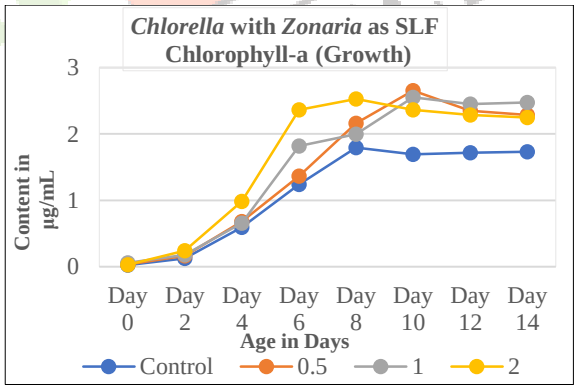
3.2 Growth Study

3.2.1 Chlorophyll-A

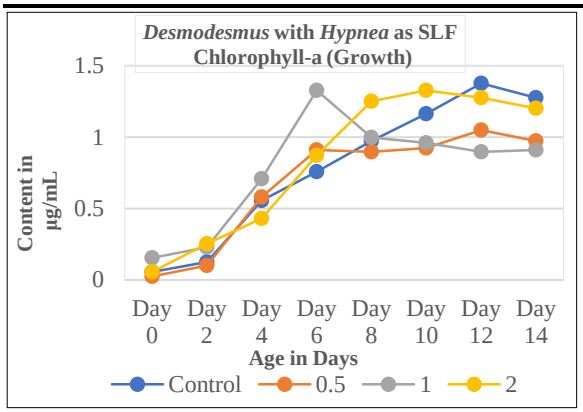
When the microalgae were treated with seaweed liquid fertilizer (SLF), chlorophyll accumulation increased significantly. The chlorophyll a content in *Chlorella* sp was highest in 2% *Hypnea* SLF (2.24814 ug/ml on day 10) **(Graph 1)** and 0.5% *Zonaria* SLF (2.6523 ug/ml on day 10) **(Graph 2)**. Maximum accumulation of 2% *Hypnea* SLF (1.32615 ug/ml on day 10) **(Graph 3)** and 1% *Zonaria* SLF (1.37667 ug/ml on day 12) **(Graph 4)** as found in *Desmodesmus sp*. Increased levels of SLF (1-2 per cent) tended to enhance chlorophyll a accumulation, a growth-promoting effect. This stimulation is associated with the presence of bioactive compounds, including auxins, gibberellins, and cytokinins, found in the SLF [30]. However, there is an inconsistent response regarding the abundance of these compounds relative to nutrient requirements and tolerance across different species.

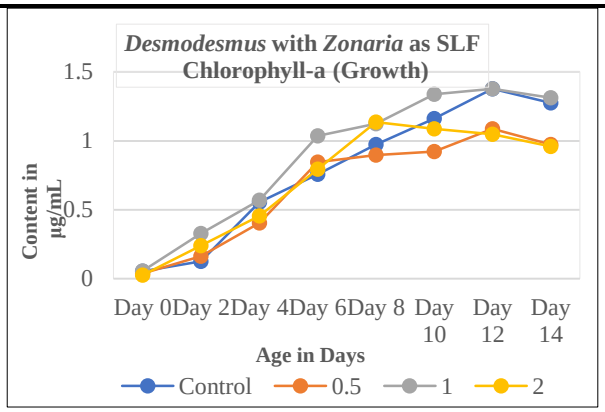


Graph 1: Growth curve of *Chlorella* with *Hypnea*.



Graph 2: Growth curve of *Chlorella* with *Zonaria*.

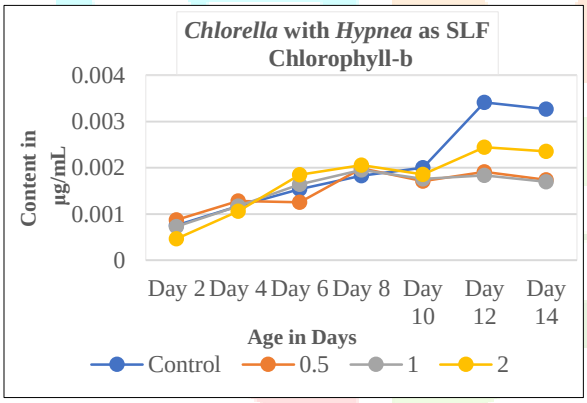


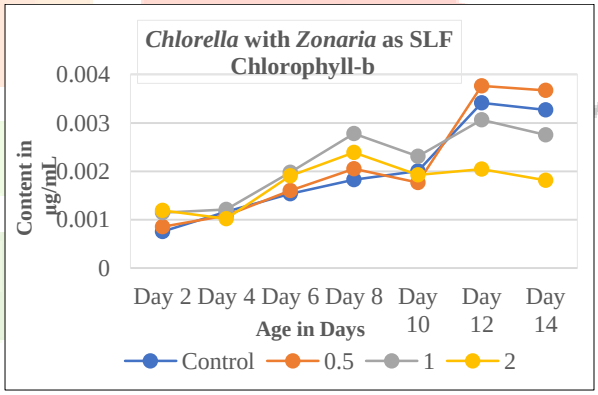


Graph 3: Growth curve of *Desmodesmus* with *Hypnea*. **Graph 4: Growth curve of *Desmodesmus* with *Zonaria*.**

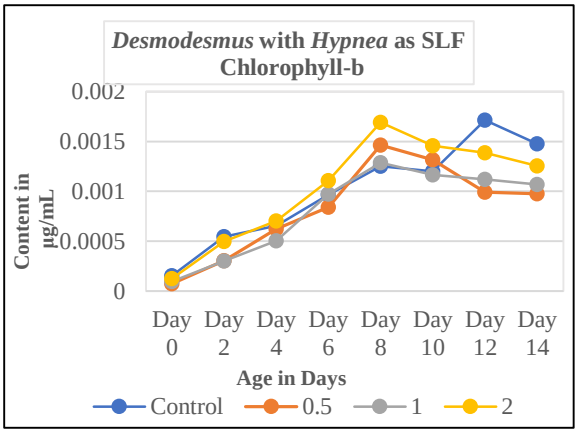
3.2.2 Chlorophyll-B

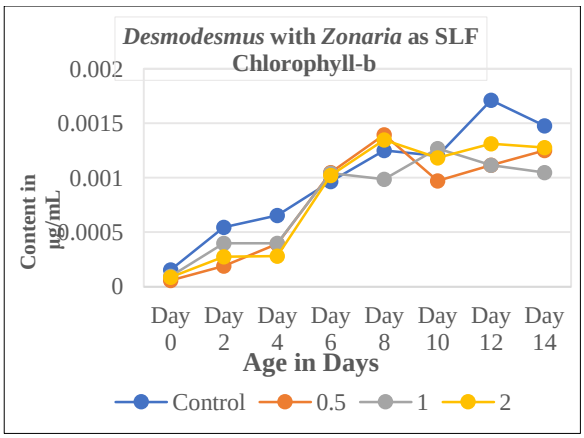
Accessory photosynthetic pigment chlorophyll b also accumulated in the microalgae when it was treated with seaweed liquid fertilizer (SLF). The maximum content of chlorophyll b was attained with 2% *Hypnea* SLF (0.002445 ug/ml on day 12) **(Graph 5)** and *Zonaria* SLF (0.003764 ug/ml on day 12) **(Graph 6)**. In *Desmodesmus* sp., 2% SLF produced the highest chlorophyll-b values of *Hypnea* (0.001692 ug/ml) **(Graph 7)** and *Zonaria* (0.001347 ug/ml) after day 8 **(Graph 8)**. These findings are consistent with previous research showing that seaweed extracts increase photosynthetic pigments by supplying phytohormones and micronutrients [2].





Graph 5: Chlorophyll-b in *Chlorella* with *Hypnea* **Graph 6: Chlorophyll-b in *Chlorella* with *Zonaria***

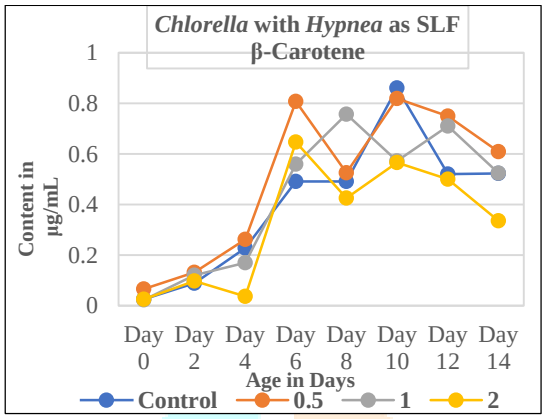


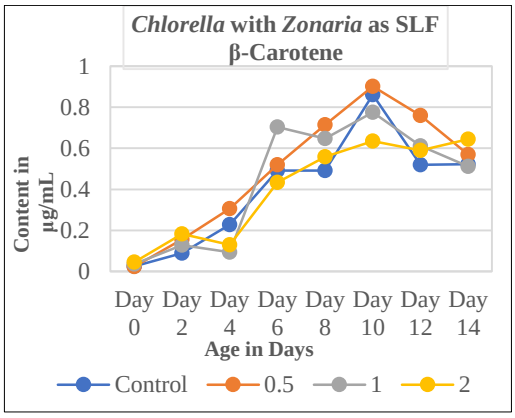


Graph 7: Chlorophyll b in *Desmodesmus* with *Hypnea*. **Graph 8: Chlorophyll b in *Desmodesmus* with *Zonaria***

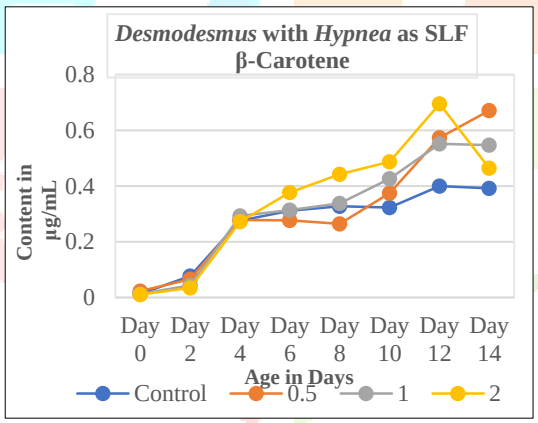
3.2.3 β-Carotene

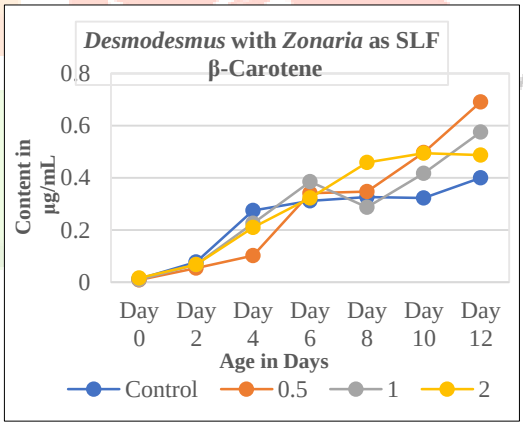
The treatment of seaweed liquid fertilizer (SLF) with β-carotene increased the amount of β-carotene in microalgae significantly. Both *Hypnea* (0.819 ug/ml) and *Zonaria* (0.901 ug/ ml) depicted maximal accumulation at 0.5% SLF in *Chlorella sp.* and 2% and 0.5% SLF in *Desmodesmus* sp, respectively **(Graph 9-12)**. These findings indicate that SLF enhances the growth and biosynthesis of carotenoids in microalgae, likely through nutrient enhancement and stress-induced stimulation of pigment synthesis, as observed previously [19].





Graph 9: **β-carotene in *Chlorella* with *Hypnea*** **Graph 10**: **β-carotene in *Chlorella* with *Zonaria.***

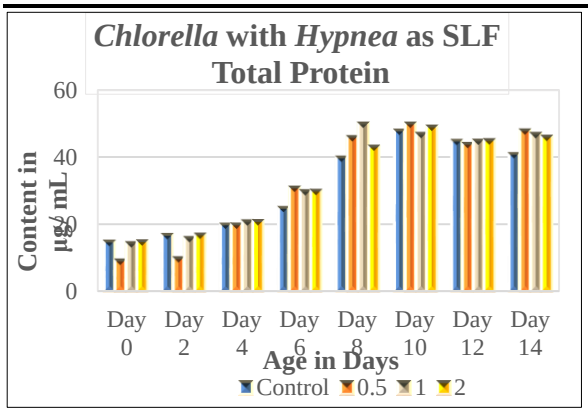


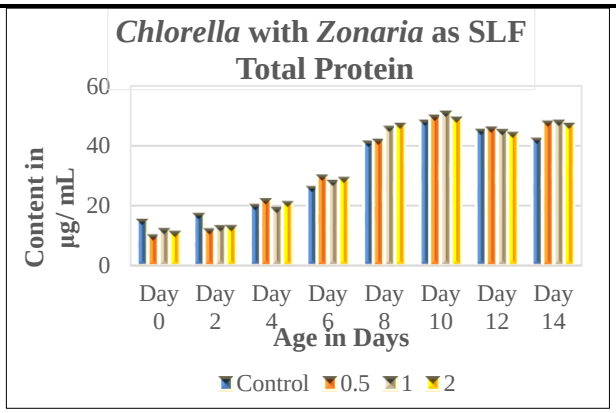


Graph 11: **β-carotene in *Desmodesmus* with *Hypnea.*** **Graph 12**: **β-carotene in *Desmodesmus* with *Zonaria.***

3. 3 Total Protein

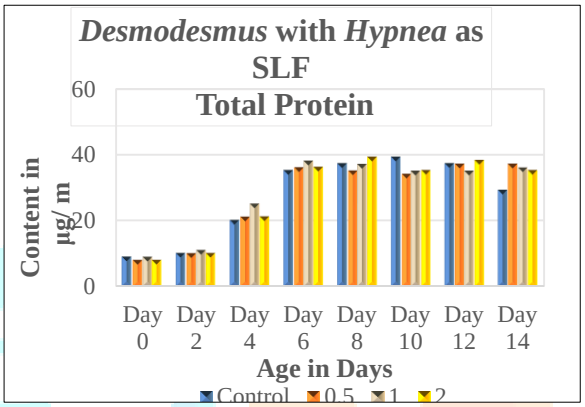
The biochemical composition of the microalgal biomass is a function of the nutrient composition of the culture medium [31]. In the current analysis, a slight increase in protein content was identified at a 1% SLF concentration for both *Hypnea sp*. and *Zonaria sp.* The maximum concentration was 50 µg/ml on the 8th and 10th days **(Graph 13-14)** for *Hypnea*-treated cultures, and 51 µg/ml on the 10th day for *Zonaria*-treated cultures. *Hypnea*-treated *Desmodesmus* sp. showed activity of 39 ug/ml at 2% concentration at the 8th day, while in *Zonaria*-treated *Desmodesmus sp*., activity of 38 ug/ml was found at 0.5% and 1% concentrations at the 12th day **(Graph 15-16)**.In accordance with Lakshmi and Sheeja (2021) [32], the same conclusion was stated through red seaweed extracts containing Chlorella vulgaris.

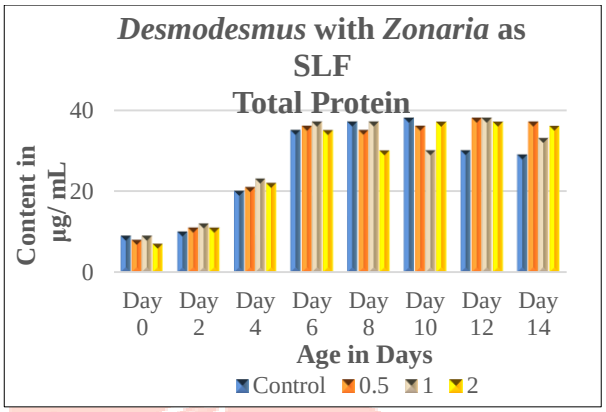




Graph 13: *Chlorella* with *Hypnea* as SLF Total protein

Graph 14 : *Chlorella* with *zonaria as SLF Total* protein



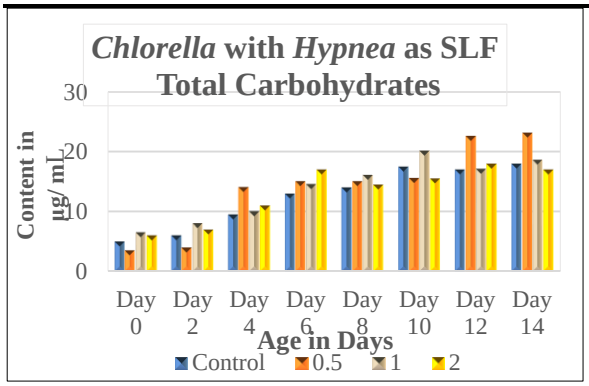


Graph 15: *Desmodesmus* with *Hypnea* as SLF Total Protein

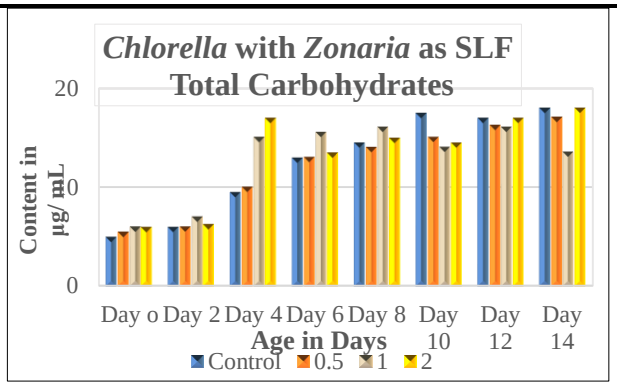
Graph 16: *Desmodesmus* with *zonaria* as SLF Total Protein

3.4 Total Carbohydrate

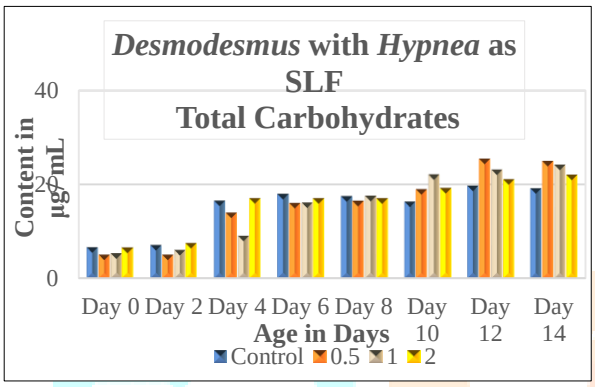
Carbohydrates, synthesized during photosynthesis, serve as essential biomolecules that regulate microalgal metabolism and growth [33,21]. In Chlorella sp., the highest carbohydrate content (22.5 µg/ml) was observed at 0.5% *Hypnea sp.* SLF on the 12th day, while 2% *Zonaria sp.* SLF showed 18 µg/ml **(Graph 17-18)** on the 14th day. In *Desmodesmus sp.*, maximum carbohydrate accumulation occurred with 0.5% *Hypnea sp.* SLF (25.5 µg/ml) and 2% *Zonaria sp*. SLF (24.5 µg/ml) on the 12th day **(Graph 19-20)**. Previous investigations [34,35] confirmed that seaweed-derived extracts can stimulate carbohydrate and glucose accumulation in microalgae



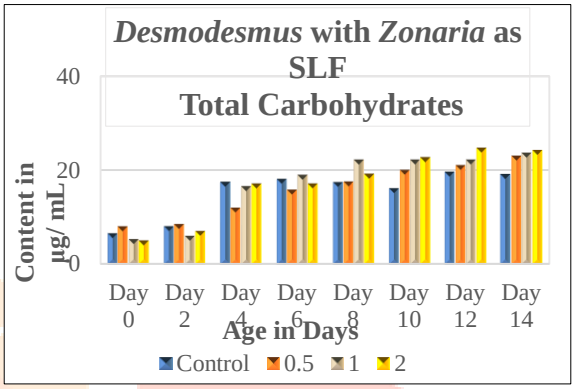
Graph 17: *Chlorella* with *Hypnea* as SLF Total Carbohydrate



Graph 18 : *Chlorella* with *zonaria* as SLF Carbohydrate



Graph 19: *Desmodesmus* with *Hypnea* as SLF Total Carbohydrate.



Graph 20: *Desmodesmus* with *Hypnea* as SLF Total Carbohydrate.

3.5 Antibacterial:

The antibacterial potential of *Hypnea sp.* and *Zonaria sp*. was evaluated using solvent extracts of ethanol, methanol, ethyl acetate, DMSO, and acetone. All extracts demonstrated significant inhibitory activity against both Gram-positive and Gram-negative bacterial strains, highlighting their broad-spectrum antibacterial potential. The maximum levels of inhibition (ZOI) were obtained against Escherichia coli (40 mm for *Zonaria sp.* and 38 mm for *Hypnea sp.* in ethanol). Comparatively, the least activity was recorded against Staphylococcus aureus (15 mm of *Hypnea sp.* and 22 mm of *Zonaria sp.* in ethyl acetate). E. coli was the most vulnerable, with B. subtilis and S. aureus being the second and third most susceptible. Such results suggest that bioactive secondary metabolites in seaweeds have antimicrobial potential, as evidenced by their effectiveness against multidrug-resistant bacterial strains in red and brown algae [25,23] **(Table 3).**

Clinical Pathogens	Ethanol				Methanol				Ethyl acetate				DMSO				Acetone			
	+ve	-ve	H	Z	+ve	-ve	H	Z	+ve	-ve	H	Z	+ve	-ve	H	Z	+ve	-ve	H	Z
Bacillus subtilis (+ve)	42	31	30	25	27	–	29	24	25	–	30	25	15	31	26	25	27	–	30	31
Escherichia coli (-ve)	40	–	38	40	40	30	30	38	40	10	35	33	36	30	30	28	40	–	33	32
Pseudomonas aeruginosa (-ve)	23	21	29	26	30	27	27	27	36	10	29	29	28	10	28	30	25	13	30	26
Staphylococcus aureus (+ve)	25	–	30	30	25	–	25	22	22	10	15	22	25	–	23	25	22	–	26	25
Klebsiella pneumoniae (-ve)	30	28	30	30	27	–	25	23	25	–	25	24	22	14	22	20	30	–	30	27

Table 3: Antibacterial Activity (H- *Hypnea* sp., Z- *Zonaria* sp..)

3.6 Antioxidant Assays

3.6.1 Dismutase of Superoxide (SOD) Activity

SOD activity differed among the vertebrates studied. *Hypnea sp*. exhibits the highest SOD activity (58.58 U/mL), whereas *Zonaria* has a notably lower value of 35.29 U/mL among macroalgae. SOD activity was significantly lower in microalgae, with both Chlorella sp. and *Desmodesmus sp.* registering 2.99 U/mL SOD activity, which means that macroalgae are more effective in their defense against oxidative stress.

3.6.2 Catalase Activity

All species have substantial catalase activity. *Hypnea sp*. displayed the best catalase, with an amount of 65.77 U/mL, and *Zonaria sp.* with a reading of 51.02 U/mL. Microalgae were also quite active, with *Chlorella sp.* and *Desmodesmus sp.* recording values of 59.10 and 63.99 U/mL, respectively. Images show significant growth rates of hydrogen peroxide.

3.6.3 Peroxidase Activity

There were slight differences in peroxidase activity. Microalgae had a little more significance (*Chlorella sp*., 5.77 U/mL; *Desmodesmus sp*., 6.88 U/mL) when compared to those of the macroalgae (*Hypnea sp*., 5.16 U/mL) and (*Zonaria sp*., 5.34 U/mL), meaning there was more dependence of peroxidase in reducing reactive oxygen species in microalgae than in macroalgae.

3.6.4 Total Proline Content

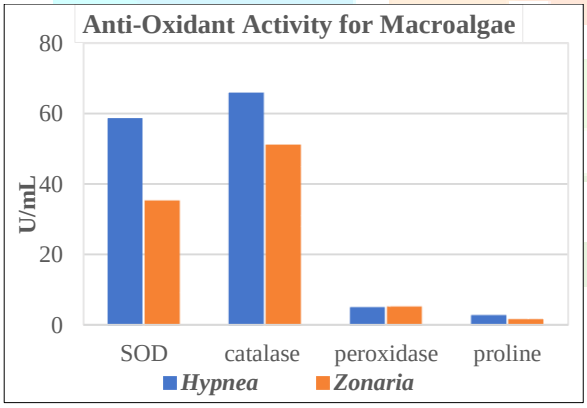
There was a great difference in the proline content across species. The proline content was highest in *Desmodesmus sp.* (9.10 U/mL) and second-highest in *Hypnea sp.* (2.95 U/mL). The smaller values were reported for *Chlorella sp.* (1.87 U/mL) and *Zonaria sp*. (1.79 U/mL), indicating a high adaptive capacity to oxidative stress in *Desmodesmus sp.*

3.6.5 DPPH Radical Scavenging Activity

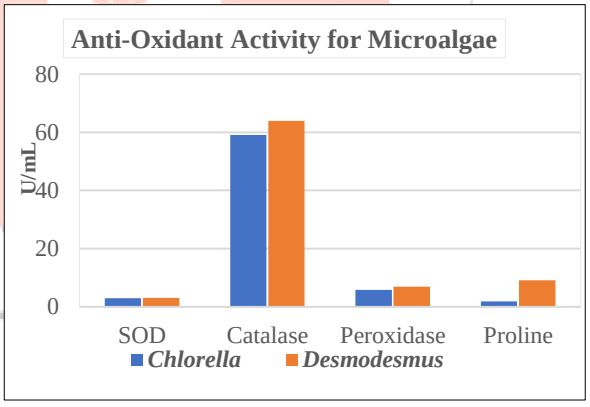
The DPPH radical scavenging activity of macroalgae and microalgae was found to be 45-50% and 41-46%, respectively. *Hypnea sp.* showed steady inhibition, and *Desmodesmus sp.* showed only a slight decrease, suggesting moderate antioxidant activity in microalgae.

3.6.6 ABTS+ Radical Scavenging Activity:

The inhibition of ABTS was better compared to DPPH in every species. Inhibition in *Hypnea musciformis* was close to 90% at 100 µg/mL, whereas in *Zonaria variegata*, the values were lower. There were moderate inhibitions of microalgae (55-65%), with a slight increase at higher concentrations.



Graph 21: Antioxidant Activity for Macroalgae



Graph 22: Antioxidant Activity of Microalgae

IV. DISCUSSION:

The present study revealed that the macroalgae *Hypnea musciformis* and *Zonaria variegata* are rich sources of secondary metabolites and, when transformed into seaweed liquid fertilizer (SLF), significantly boost microalgal growth, pigments, and biochemical composition. The phytochemical screening showed the presence of alkaloids, saponins, tannins, flavonoids, glycosides, and terpenoids in both macroalgae, which agrees with previous studies that marine algae are useful sources of bioactive compounds with antioxidant and antimicrobial properties [36]. The presence of macronutrients, trace minerals, and natural phytohormones such as auxins, cytokinins, and gibberellins, which induce photosynthetic pigment synthesis and cellular metabolism, is believed to be responsible for the growth-promoting effect of SLFs [37]. These phytohormonal components increased chlorophyll a, chlorophyll

b, and β-carotene content, indicating higher photosynthetic ability and biomass production under the SLF condition.

The increased biochemical improvement in protein and carbohydrate content suggests that SLF-mediated nutrient uptake and assimilation were successful, thereby promoting nitrogen uptake and carbon fixation pathways in the microalgae, consistent with an earlier report on seaweed-derived biostimulants [38]. Apart from that, the macrolgal extracts showed strong antibacterial activity against both Gram-positives and Gram-negatives, including *Escherichia coli* and *Bacillus subtilis*, as in a previous study on *H. musciformis*, with large inhibition zones against many bacterial strains [39]. The enhanced profiles of antioxidant enzymatic activities, especially SOD and catalase, combined with high radical-scavenging potentials (DPPH and ABTS assays), clearly demonstrate an efficient mechanism of oxidative-stress scavenging by the polyphenolic and flavonoid constituents of the extracts [40].

Taken together, these findings validate that *H. musciformis* and *Z. variegata* can be used as environmentally friendly bioresources, as they have dual functions: promoting microalgal growth and producing potent antimicrobial and antioxidant activities. So, their application as seaweed-derived fertilizers would thus enhance algal productivity and, at the same time, provide biologically active compounds with agricultural, aquacultural, and pharmaceutical applications.

V. CONCLUSION:

The current research has revealed that Hypnea musciformis and Zonaria variegata are potential sources of bioactive substances with important functional properties. The reported phytochemical and secondary metabolite activities of the extracts underscore the usefulness of various solvents in extracting multiple bioactive compounds. The growth of microalgae was improved by incorporating these seaweed extracts as seaweed liquid fertilizer (SLF), which increased their pigment content, protein and carbohydrate levels, and antioxidant and antibacterial activity. These findings suggest the potential to enhance the nutritional value of microalgae by using seaweed extracts as foodstuffs and biotechnological products. Future research is needed to isolate, identify, and characterize the bioactive compounds that induce these positive effects.

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