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Growth and Biochemical Characteristics of Microalgae *Chlorella vulgaris* Grown on Various Combinations of Fish Waste Hydrolysate and Seaweed Hydrolysate

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ABSTRACT

Background: to utilize the fish waste and seaweed as a media for the growth of microalgae *Chlorella vulgaris* and carbon sequestration.

Methods: Culture of Chlorella vulgaris in the fish waste and seaweed hydrolysate, analysed the cell density, biomass, chlorophyll, carotenoid content, carbon-di-oxide sequestration and proximate of microalgae (Protein, Carbohydrate, Lipid and Ash).

Result: In the combination of fish acid hydrolysate and *sargassum sp*., acidhydrolysate, the respective peak values of cell density, average specific growth rate, biomass, chlorophyll a and b, carotenoid contentand carbon-di-oxide sequestrated recorded are $4.26\pm0.06\times10^6$ cells/ml, 0.58 µ/day, 1.98±0.04 g/l, 5.59±0.09 µg/ml and 3.69±0.04 µg/ml, 3.64±0.05 µg/ml, 3.73±0.07 g/l/d in 3:6 combination. Corresponding protein, carbohydrate, lipid and ash content observed in the resultant algae are 51.77±0.6%, 15.4±0.2%, 10±0.53 and 7.08±0.4% respectively.

Key words: *Chlorella vulgaris*, Fishwaste, Hydrolysate, Microalgae, Seaweed.

INTRODUCTION

Climate change and energy crisis are prime concern of environmentalists of this century. It has been addressed by terrestrial plants. But the algae in aquatic systems can consume nearly 34 gigatons of carbon (Paul *et al.,* 2014). While planting trees in all possible means is estimated to reduce nine gigatons that too at a cost of displacing a sizable land meant for farming. Growing algae would simultaneously resolve energy crisis climate change and human health.

Algae are photosynthetic organisms that can thrive in wide range of habitats including lakes, ponds, rivers, oceansand even wastewater. They also have the ability to tolerate wide range of temperatures, salinities, pH values and light intensities. Microalgae also play an important role in CO_2 sequestration, as the CO_2 needed for their photosynthetic metabolism. It was estimated that 1 kg of dry algal biomass consumes about 1.83 kg of CO_2. It has a wide range of application in biofuels, health supplements, pharmaceuticals, cosmetics production and also in application in wastewater treatment and atmospheric CO_2 mitigation (Das *et al.,* 2011). *Chlorella vulgaris* contains a significant amount of proteins, carbohydrates, lipids, vitamin C, β-carotenes and B vitamins (B_1, B_2, B_6 and B_{12}), which is why it is commonly used for the preparation of food supplements, as well as for the production of cosmetics, clinical treatments and even for the detoxification of heavy metals in wastewater. Algal production gained a global attention due to its potential growth, the formation of large amounts of biomass, less land requirement, high oil accumulation and feasible CO_2 sequestration, (Quinn and Davis, 2015). Algal cultures also produce sustainable product towards aquaculture feeds, human food supplements, pharmaceuticals and biofuels. Due to the algae merits considering for cultivation, wherein proper culture condition including nutrient media play a pivotal role and there is a need to formulate various growth media which allow greater control of growth and easy scaling for high productions at the industrial level that cover the current global needs (Coronado-reyes *et al.,* 2020). The goal of this study has been designed to create a low cost nutrient medium using fish waste and seaweed for the growth medium in order ensure the fairly high growth of *Chlorella* organic media.

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MATERIALS AND METHODS

Collection of fish waste

Fish market wastes were procured from Pazhaverkadu fish landing center. The fish mainly consisted of heads, tails, gills, fins and viscera of various fishes. The collected fish wastes were washed with tap water, minced and stored at -4°C until further used.

Preparation fish waste acid hydrolysate

Acid Fish hydrolysis was performed according to the protocol prescribed by Wisuthiphaet *et al*. (2014). The raw material, minced fish stored at - 4°C was thawed to room temperature. Two kg of this minced fish was mixed with one liter distilled water. From this mix of fish solution 50 ml is taken and added with 4M HCl to arrive at a pH of 4. This mix was subjected to 121°C for 90 minutes at 15 psi to carry through acid hydrolysis. This solution was allowed to cool and the hydrolysis reaction was terminated by elevating the pH to 5 by adding 6M, NaOH solution. This solution was partially filtered to remove huge pieces of bonesand stored at 4°C for further use.

Preparation seaweed acid hydrolysate

The sargassum seaweed was collected and washed with freshwater to remove sand and dirt. It was dried in hot air oven at 55°C for 72 h. The dried seaweed was ground into fine powder. Seaweed acid hydrolysate was prepared as described by Sarkar *et al.* (2018). 100 g of both the powdered sea weeds were mixed with 1000 ml of 1% HCl and sterilized for 10 min. The mixture was cooled and filtered using cheese cloth.

Proximate compositions evaluation

Proximate composition, *i.e.* moisture, crude protein, crude fat, ash, potassium and phosphorous of prepared fish waste manure and seaweed manure were determined as per standard methods of AOAC (2005), Priyatharshni *et al*., (2024) Ruby *et al*. (2022).

Culture of microalgae

The prepared fish waste and seaweed manure were sterilized in an autoclave at 121°C for 20 min and cooled to room temperature prior to use. The fish waste and seaweed manure stock solution was diluted with sterilized fresh water to derive 3%, 6%, 9%, media. BBM mediumwas kept as the control medium for *Chlorella vulgaris,* to compare the growth and biochemical characteristics (Joshna *et al*., 2024). The cultivation of algae was carried out in 3 L transparent plastic containers at room temperature and placed under white fluorescent light (light intensity of 2500 lux) with a light: dark cycle of 12:12 h. An amount of 10% inoculum containing 4.36×10^6 cells/ml *Chlorella vulgaris* was added. Triplicate cultures were made for all the treatments. The fish manure was Fish waste acid hydrolysate (F1) and seaweed manure was *Sargassum* acid hydrolysate (S1). Their combination represents D1 (F1 3% + S1 3%), D2 (F1 3% + S1 6%), D3 (F1 3% + S1 9%), D4 (F1 6% + S1 3%), D5 (F1 6% + S1 6%), D6 (F1 6% + S1 9%), D7 (F1 9% + S1 3%), D8 (F1 9% + S1 6%) and D9 (F1 9% + S1 9%).

Estimation of chlorophyll and carotenoid

Chlorophyll (a andb) and carotenoid was estimated according to Arnon (1949) and Kirk and Allen, (1965) respectively. 20 mL of the culture was centrifuged at 10,000 rpm for 10 min. The collected pellet was mixed with 90% acetone and refrigerated for 12 h covered with aluminium foil. The mixture was centrifuged at 5000 rpm for 10 min. the supernatant was measured using UV-spectrometer at the absorbance value of 480 nm, 663 nm and 645 nm.

Chlorophyll a (µg/ml) = 12.7 (A663)-2.69 (A645)

Chlorophyll b (µg/ml) = 22.9 (A645)-4.68 (A663)

Carotenoids (µg/ml) = A480 + (0.114×A663) % (0.638×A 645).

Where,

A = Absorbance at respective wave length.

Estimation of carbon dioxide assimilation

$$CO_2 \text{ fixation rate} = 1.88 \times P$$

Where,

P is the overall biomass productivity in (g $L^{-1}d^{-1}$) (Barahoei *et al.,* 2020). The all above parameters were estimated at three days intervals.

Estimation of proximate composition of algae

The cell concentration of each sample was measured by counting the cell number every third day. The cell density was counted by Haemocytometer for *Chlorella vulgaris*. The biomass content in the culture was calculated at the end of cultivation (21 days). For the analysis of various biochemical factors such as protein, carbohydrate and lipid, 100 mL algal sample from each concentration of mass-cultured alga was centrifuged at 10,000 rpm for 10 min at 4°C. The residue was again centrifuged with distilled water and the content was filtered through 1 mm pore size GF/F filter paper. The total protein of the sample was analyzed using Lowry's method (Lowry *et al*., 1951). Total carbohydrate was estimated using the phenol sulfuric acid method (Dubois *et al*., 1956). Total Lipid was estimated using the modified Barnes and Blackstock (1973) method. Ash content was analyzed using the procedure (AOAC, 2005), Priyatharshni *et al*. (2023).

Statistical analysis

The observed and arrived at data have been subjected to one-way ANOVA analysis followed by Duncan tests using statistical software SPSS version 22 (SPSS Inc., Chicago, USA). The values with the probability less than P< 0.05 were considered significant.

RESULTS AND DISCUSSION

Proximate manure value of fish waste and seaweed liquid manure

Fish liquid manure would be a good potential source for animals in arid regions as it contained high protein (Al-Abri *et al.,* 2014), reflecting the nitrogenous ingredients.The nutrient density of fish derivatives has given a picture of nitrogen percentages of 2.31 values are in Table 1, in accordance with the values (fish acid hydrolysate 2.5%) of Wisuthiphaet *et al*. (2014). The higher percentages of acid hydrolysis is due to the role of acid in retaining a higher percentage of ammoniacal form of nitrogen, than alkaline environs (Klimczyk *et al.,* 2021) and breakdown of nitrogenous fractions to highly available form of nitrogen. Reduction of protein content in the ensilage may be due to break down of protein (FAO, 2007). In the present study the protein content of FWH is higher than the earlier reports on fish hydrolysates (Sahu *et al.,* 2011). Phosphorous is the most limiting the factor of phytoplankton (Kumar *et al.,* 2012) and even a little spike would enhance the microalgae production (Van Mooy *et al.,* 2009), remarkable on fulfilling limitations. This phosphorous value coincides with the value of Alvarado *et al*. (2008) and Sahu *et al.* (2011). The same trend of potassium 0.11% I fish acid hydrolysate was observed. The minerals as ash content were more in acid and alkaline hydrolysates, keeping more of minerals, (3.98%) in suspension in fish hydrolysate (Shu and Tsai, 2016).

The nitrogen (1.93%), phosphorous (0.02%), potassium (1.94%) and ash content (5.02%) shown in Table 1 of acid hydrolysates of *Sargassum* sp., was found to be congruent with earlier investigations of Uthirapandi *et al.,* (2018), Malik *et al.,* (2018).

Cell density estimates of *Chlorella vulgaris*

In 21 days of Chlorella culture, fish and seaweed combination, a ratio of 3: 6 (fish waste: seaweed), provided optimal nutrients for climax performance, in terms of cell density (4.26×10^{6} cells/ml), remarkably higher than control (2.95×10^{6} cells/ml), the values are shown in Table 2. Surprisingly, it is noted that higher concentrations of these ingredients shown a minimal cell count, probably due to impeding light. Hence batch harvest with periodic nutrient replenishment strategy would greatly serve in maximizing the harvest. The lowest cell density was observed in D7 (9:3), D8 (9:6), D9 (9:9) fish waste and seaweed manure concentrations, the lower concentrations is best suited for the culture of microalgae. The higher concentrations shows lower growth may be due to the initial development of colour coupled with turbidity of suspended particulates resisted the light penetration and thereby inhibiting the photosynthesis (Hena *et al*., 2021).

Biomass content in *Chlorella vulgaris*

In *Chlorella vulgaris* culture, of the various combinations tried for biomass production, concordantly 3% of fish waste

Table 1: Composition of fish waste and seaweed liquid manure.

Components (%)	FW(Ac.H)	S(Ac.H)
Nitrogen	2.31±0.06^{ab}	1.93±0.02^{b}
Phosphorous	0.09±0.01^{a}	0.03±0.00^{a}
Potassium	0.11±0.00^{a}	1.94±0.03^{b}
Ash	3.48±0.33^{b}	5.02±0.24^{c}
Moisture	83.6±2.09^{c}	85±2.00^{d}

Values were mean±SD. Superscript in each column showed the significance difference (p<0.05). FW(Ac.H)- Fish waste Acid hydrolysate,S(Ac.H)- *Sargassum* Sp., acid hydrolysate.

Table 2: Cell density of *Chlorella vulgaris* - fish waste acid hydrolysate and *Sargassum* sp., acid hydrolysate.

Days	Cell density ($\times10^{6}$ cells/ml)									
	Control	D1	D2	D3	D4	D5	D6	D7	D8	D9
0	0.3±0.02^{a}	0.3±0.02^{a}	0.3±0.02^{a}	0.3±0.02^{a}	0.3±0.02^{a}	0.3±0.02^{a}	0.3±0.02^{a}	0.3±0.02^{a}	0.3±0.02^{a}	0.3±0.02^{a}
3	0.44±0.03^{bc}	0.52±0.04^{c}	0.75±0.06^{d}	0.75±0.03^{d}	0.52±0.03^{c}	0.44±0.04^{bc}	0.44±0.09^{bc}	0.36±0.05^{a}	0.30±0.02^{a}	0.33±0.01^{a}
6	1.45 ±0.02^{de}	0.88±0.03^{c}	2.44±0.09^{fg}	1.40±0.12^{d}	1.59±0.02^{ef}	1.54±0.11^{e}	0.78±0.02^{c}	0.65±0.07^{b}	0.45±0.03^{a}	0.54±0.02^{ab}
9	1.91±0.09^{d}	1.19±0.10^{c}	2.49±0.09^{f}	2.22±0.18^{e}	2.14±0.14^{e}	1.90±0.07^{e}	0.89±0.03^{b}	1.28±0.05^{d}	0.55±0.02^{a}	0.64±0.02^{a}
12	2.46±0.22^{e}	1.36±0.03^{c}	2.96±0.01^{f}	2.54±0.10^{e}	2.62±0.12^{e}	2.63±0.14^{e}	1.04±0.06^{b}	1.58±0.09^{d}	0.66±0.05^{a}	0.63±0.04^{a}
15	2.60±0.41^{d}	1.62±0.07^{c}	3.53±0.07^{f}	3.09±0.12^{e}	2.87±0.12^{e}	2.94±0.07^{e}	1.20±0.10^{b}	1.84±0.09^{c}	0.57±0.02^{a}	0.49±0.03^{a}
18	2.78±0.13^{d}	2.03±0.03^{c}	3.95±0.05^{h}	3.76±0.11^{g}	3.18±0.18^{e}	3.48±0.21^{f}	1.35±0.31^{b}	1.80±0.10^{c}	0.57±0.02^{a}	0.49±0.03^{a}
21	2.95±0.06^{e}	2.63±0.06^{d}	4.26±0.06^{h}	4.10±0.12^{g}	4.13±0.12^{gh}	3.86±0.13^{f}	1.64±0.08^{c}	1.49±0.04^{b}	0.65±0.03^{a}	0.56±0.05^{a}

Values were mean± SD. Superscript in each column showed the significance difference (p<0.05).

and 6% of seaweed liquid manure has rendered favourable nutritional base for maximal production, almost 50% over and above control in most of the cases. A remarkable biomass production of 400 mg/L was achieved at day 12 by Alazaiza *et al*. (2023) in Chlorella vulgaris in sewage water. Similar observation found in Kumar *et al.* (2012) and Kumaran *et al.* (2023) in the culture of Chlorella vulgaris in sewage waste water and palm oil mill effluent respectively. The decrease in the biomass was observed at higher concentrations such as in D7, D8 and D9 due the suspended particles and attributed due to turbidity, which decreased light intensity and inhibited the accretion of biomass (Bohutskyi *et al.,* 2016).

Chlorophyll content in *Chlorella vulgaris*

Chlorophyll *a*, the primary photosynthetic pigment and chlorophyll *b*, the accessary photosynthetic pigment, evaluated in chlorella cultivation. 3% of fish and 6% of seaweed combinations showed the best chlorophyll a and b content of 5.59 µg/ml and 3.69 µg/ml respectively. The values are shown in Table 3. The chlorophyll content made out here synchronised values of chlorophyll content found in Chlorella culture in Palm oil Mill effluent by Kamyab *et al.* (2019), He *et al.* (2023) in aquaculture waste water, Wu *et al.* (2023) in secondary effluent water.

Carotenoid content of *Chlorella vulgaris*

The precursor of vitamin A, beta carotene represented in total terms as Carotenoid is well packed in Chlorella. The combo with 3% fish and 6% seaweed (3.64 µg/ml).The values are shown in Table 3. Carotenoid serves as accessory pigment for photosynthesis and also as an antioxidant that may reduce damage to the cell, ribonucleic acid and deoxyribonucleic acid. Lu *et al.* (2015) demonstrated that the microalgae *Chlorella* sp., grow well in wastewater as well as in the nutrient medium. Increased production of carotenoids in presence of add on carbon source may have a say in the culture (Velichkova *et al.,* 2014). These values were coinciding with *chlorella vulgaris* culture of municipal waste water (Singh *et al.,* 2022).

Carbon-di-oxide sequestration of *Chlorella vulgaris*

Addressing climate change has become a priority subject of any enterprise. Algal probability of abstracting this carbon dioxide is widely understood, but least emplaced in pragmatic terms. Fish waste at 3% and seaweed at 6%

Table 3: Biomass, chlorophyll, carotenoid and carbo-di-oxide sequestration of *Chlorella vulgaris* - fish waste acid hydrolysate and *Sargassum* sp., acid hydrolysate at the end of 21 day culture period.

	Biomass (g/l)	Chlorophyll-a (µg/ml)	Chlorophyll-b (µg/ml)	Carotenoids (µg/ml)	CO2 sequestration (g/l/d)
Control	1.16±0.04^{d}	3.58±0.17^{e}	2.69±0.09^{e}	2.48±0.1^{e}	2.19±0.07^{d}
D1	1.16±0.04^{d}	3.2±0.06^{d}	2.5±0.03^{d}	2.31±0.03^{d}	2.18±0.07^{d}
D2	1.98±0.04^{g}	5.59±0.09^{h}	3.69±0.04^{h}	3.64±0.05^{h}	3.73±0.07^{d}
D3	1.89±0.08^{f}	5.27±0.26^{g}	3.53±0.13^{g}	3.46±0.15^{g}	3.55±0.15^{f}
D4	1.91±0.08^{f}	5.14±0.12^{g}	3.47±0.06^{g}	3.39±0.07^{g}	3.58±0.15^{f}
D5	1.73±0.08^{e}	4.78±0.11^{f}	3.29±0.05^{f}	3.19±0.06^{f}	3.26±0.16^{e}
D6	1.04±0.05^{c}	2.77±0.11^{c}	2.29±0.05^{c}	1.97±0.06^{c}	1.95±0.1^{c}
D7	0.94±0.03^{b}	2.52±0.03^{b}	2.16±0.01^{b}	1.83±0.01^{b}	1.77±0.05^{b}
D8	0.41±0.02^{a}	1.71±0.08^{a}	1.76±0.04^{a}	1.39±0.04^{a}	0.77±0.04^{a}
D9	0.35±0.03^{a}	1.63±0.12^{a}	1.72±0.06^{a}	1.34±0.07^{a}	0.67±0.07^{a}

Values were mean±SD Superscript in each column showed the significance difference ($p<0.05$).

Table 4: Proximate composition of *Chlorella vulgaris* fish waste acid hydrolysate and *Sargassum sp*., acid hydrolysate.

	Total protein (%)	Total carbohydrate (%)	Total lipid (%)	Ash content (%)
Control	45.1±0.72^{a}	11.33±0.31^{b}	5.29±0.24^{a}	2.29±0.25^{a}
D1	49.93±0.45^{d}	12.15±0.05^{c}	7.51±0.45^{c}	4.21±0.06^{c}
D2	51.77±0.6^{f}	15.4±0.2^{f}	10±0.53^{f}	7.08±0.4^{f}
D3	50.77±0.67^{e}	13.5±0.36^{d}	8.52±0.43^{d}	5.62±0.12^{d}
D4	51.58±0.13^{f}	14.77±0.23^{e}	9.59±0.25^{ef}	6.49±0.19^{ef}
D5	49.9±0.1^{d}	12.37±0.38^{c}	9.24±0.55^{e}	6.34±0.4^{e}
D6	48.44±0.35^{c}	12.37±0.35^{c}	8.17±0.15^{d}	5.27±0.09^{d}
D7	47.33±0.21^{b}	11.33±0.06^{b}	6.93±0.42^{c}	3.83±0.5^{c}
D8	48.48±0.38^{c}	10.99±0.1^{b}	6.23±0.06^{b}	3.13±0.42^{b}
D9	46.63±0.41^{b}	10.4±0.4^{a}	5.84±0.07^{ab}	2.74±0.32^{b}

Values were mean±SD. Superscript in each column showed the significance difference ($p<0.05$).

levels provided adequate nutrients in generating bio stuff fixing sizeable carbon in it scrubbed the carbon-di-oxide of 3.64 g/l/d. CO_2 capture, by culturing the microalgae in waste water, is now reckoned to be an environmentally sustainable and economically viable option (Jain *et al.,* 2019). *Chlorella* sp. can tolerate the pollutant load and has been proven to be a suitable species for CO2 fixation (Hariz *et al.,* 2018). The CO_2 sequestration in *Chlorella* sp. has been widely addressed with values, 1.11g/l/d (Pourjamshidian *et al.,* 2019), 1.2 g/l/d (Cheng *et al*., 2019). The current study stands perspective of carbon fixation, making organic cultivation of chlorella as ideal choice, giving a lead to carbon trading probability. The values are shown in Table 3.

Proximate composition of *Chlorella vulgaris*

The combination of 3% fish waste and 6 % (D2) seaweed showed the greater protein content of 51.7%, Carbohydrate (15.4%), lipid (9.6%) and ash content (6.27%) show in Table 4. The protein, carbohydrate and lipid content in chlorella have not shown much of variation among the treatments. Generally *Chlorella* Sp., consists of 48% Protein, 16% lipid, 10% Carbohydrate, (Kafyra *et al.,* 2018). Arora and Philippidis, (2021) found that the biomass concentration is 2.83 g/L consisting of 34% lipids and 26% carbohydrates in chlorella vulgaris cultured in sweet sorghum bagasse hydrolysate. The mineral content of the current study appears to be fair than represented (4.4%) by Agwa *et al.* (2014), (4.5%) by Zakaria *et al.* (2017).

CONCLUSION

The present experiment was aimed to study the growth of *Chlorella vulgaris* in an organic medium composed of fish waste and seaweed manure. This study was done by evaluating various combinations of fish waste and seaweed manure in supporting the growth, biochemical parameters and carbon sequestration potential of *Chlorella vulgaris.* Among the various combination of fish waste and seaweed manure, the combination of 3% fish acid hydrolysate and 6% *sargassum* sp., acid hydrolysate was found to be better combo for the culture of *Chlorella vulgaris* in terms of cell density, biomass and biochemical parameters such as protein, carbohydrate and lipids. Organic cultivation of microalgae *Chlorella vulgaris* is of great economic value and carbon sequestration in climate change.

Conflict of interest

There is no conflict of interest

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