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Lignite humic acid for enhanced hatchery shrimp seed production by improving phytoplankton growth

S. Nancy Catherine[1], S.T. Somasundaram[1]*, R. Shanmugasundaram[2] , V. Manoharan[2]
[1]Centre of Advanced Study in Marine Biology, Faculty of Marine Sciences,
Annamalai University, Parangipettai – 608502, Tamil Nadu, India.
[2] Centre of Advanced Research and Development, Neyveli Lignite Corporation India Limited, Neyveli– 608508, Tamil Nadu, India.
*Corresponding Author: drstscasau@gmail.com

Abstract: *Lignite resource is abundant and occupies an important position of the coal reserves in our country. The processing of lignite is very extensive and it includes extraction of humic acid as by-product. Since humic acid stands as one of the potential organic resources, it has broad utilization prospect in the fields of agriculture, medicine, environment protection and industry. Centre of Advanced Research and Development, Neyveli Lignite Corporation India Limited (CARD - NLCIL), Neyveli, periodically supplied humic acid for testing the efficiency of phytoplankton growth. Humic acid proved highly significant positive effect on the growth of marine phytoplankton cultures of Nannochloropsis sp., Tetraselmis sp., Thalassiosira pseudonana and freshwater phytoplankton culture of Spirulina sp. It was also observed that humic acid has negative effect on the growth of Dunaliella salina. This study revealed high yield of phytoplankton production when compared to the culture grown in conventional medium. Humic acid had an optimum stimulating effect on the growth of phytoplankton at lower concentration. All our experiments demonstrated that humic acid is a very effective enhancer to improve the large-scale cultivation of phytoplankton species. T. pseudonana is a vital candidate species in shrimp hatchery as it is used as a suitable nutritional dietary live feed. Our experimentation in T. pseudonana growth with humic acid showed 20 folds increase in biomass and it also exhibited improved growth and survival rate of Litopenaeus vannamei larvae. From the study, it is found that humic acid helps in the growth of phytoplankton at its specific concentration and thereby it could be used as a low-cost and high-yield investment for the better aquaculture production.*

Key Words: *marine, phytoplankton, humic acid, aquaculture, shrimp.*

1. INTRODUCTION:

In India, due to the fast rate of urbanization, the only dependable source of mankind that remains for the nutritive supply is huge unknown potential of aquatic resources. Aquaculture is one of the flourishing industries in Indian perspective and it largely depends on shrimp and fish. Owing to enormous commercial potential there is intensive and highly competitive culture practices to increase the number of harvests and enhance the number of animals per harvest [1]. The aquaculture production is improved through phytoplankton as a result of several attempts of their culture practices. The population of phytoplankton in any aquatic system is the biological wealth of water as they play a crucial role in stabilizing the water quality, nutrition supply and microbial control [2, 3]. To this point, our study scopes out the conversion of lignite humic acid into an inducer for the growth of phytoplankton and directs the feed supplement for aquaculture. Humic acid is a major constituent present majorly in soil, peat and coal. It is produced by biodegradation of dead organic matter and serves as natural organic fertilizer by improving the production of phytoplankton. It also functions as stimulating agent that enhance the permeability of cell membrane and increase the uptake of nutrients from the medium. Humic acid supplemented phytoplankton generates increasing range of secondary producers thereby levelling the ecological balance within the ecosystem by serving a good nourishing fodder to culture commercialized aquatic animals. It has been demonstrated that humic acid stimulate the growth of various phytoplankton but the mechanism behind this growth stimulation have not been clarified yet. It is assumed that addition of humic acid is growth promoting just as inorganic nitrogen is and also act as chelators enhancing trace element availability to phytoplankton. Thus the study explored the possibilities of using humic acid as a nutritional supplement to enhance the phytoplankton production which in turn will increase the growth of pond reared aquaculture animals.

2. METHODOLOGY:

Phytoplankton sampling and analyses:

Phytoplankton were collected using standard plankton net of 1m length, 25cm mouth diameter, and

mesh size of 20µm. The plankton net was towed horizontally to sample the phytoplankton. For horizontal towing, plankton sample was collected by lowering the net horizontally into the water then pulled until the net extended and began to tow. The net was pulled straight up through the water column. The samples were then rinsed into collection vessels and brought to the laboratory for phytoplankton identification and it was identified based on the morphological illustrations given by [4].

Selection of phytoplankton:

Phytoplankton species have been selected on the basis of their mass-culture potential, cell size and fast doubling rate with minimal requirement of medium. As marine phytoplankton cultures of *Nannochloropsis* sp., *Tetraselmis* sp., *Dunaliella salina, Thalassiosira pseudonana* and freshwater phytoplankton culture of *Spirulina* sp. exhibited the required characters they were selected for experimentation.

Maintenance of cultures:

The water samples were collected from Vellar estuary, Parangipettai (Coordinates: 11°29'N and 79°46'E) and it was serially diluted in sterile f/2 medium. By microscopic examination, *Nannochloropsis* sp., *Tetraselmis* sp., *Dunaliella salina, Thalassiosira pseudonana* and *Spirulina* sp. were isolated maintained as a axenic culture and used for further experiments. Stock cultures were maintained in Elrenmeyer flasks at a light intensity of 1000lux and a temperature of 18±1°C. Constant illumination is required for the maintenance. Stock cultures can be stored for about a month and then transferred to create a new culture line.

Media preparation:

Seawater used for culturing phytoplankton should be made free of organisms that may compete with the growth of desired phytoplankton. Sterilization of the seawater by the physical method such as filtration and autoclaving was performed. Autoclaving (15 to 45min. at 120°C and 20psi, depending on the volume) or pasteurization (80°C for 1-2h) is mostly applied for sterilizing the culture medium in test tubes, Erlenmeyer and carboys.

f/2 medium composition:

f/2 medium is a common and widely used general enriched seawater medium designed for culturing marine phytoplankton. The concentration of the original formulation termed "f Medium" has been reduced by half according to [5]. f/2 medium was prepared by the addition of the following components to 950ml of filtered natural seawater. The final volume was brought to 1litre with filtered natural seawater. Then it was autoclaved.

Table 1: Composition of f/2 medium

Component	Stock solution	Quantity	Molar concentration in final medium
$NaNO_3$	75 g/L $d.H_2O$	1ml	8.82×10^{-4} M
$NaH_2PO_4 .H_2O$	5 g/L $d.H_2O$	1ml	3.62×10^{-5} M
$Na_2SiO_3 .9H_2O$	30 g/L $d.H_2O$	1ml	1.06×10^{-4} M
Trace metal solution	as mentioned in table 2	1ml	-
Vitamin solution	as mentioned in table 3	0.5ml	-

Table 2: Composition of Trace Metal Solution

Component	Stock solution	Quantity	Molar concentration in final medium
$FeCl_3 .6H_2O$	-	3.15g	1.17×10^{-5} M
$Na_2EDTA .2H_2O$	-	4.36g	1.17×10^{-5} M
$CuSO_4 .5H_2O$	9.8 g/L dH_2O	1ml	3.93×10^{-8} M
$Na_2MoO_4 .2H_2O$	6.3 g/L dH_2O	1ml	2.60×10^{-8} M
$ZnSO_4 .7H_2O$	22.0 g/L dH_2O	1ml	7.65×10^{-8} M

$CoCl_2 .6H_2O$	10.0 g/L dH_2O	1ml	4.20×10^{-8} M
$MnCl_2 .4H_2O$	180.0 g/L dH_2O	1ml	9.10×10^{-7} M

For the preparation of trace metal solution, the components given below were added to 950ml of distilled H_2O and brought to the final volume of 1litre with distilled H_2O and autoclaved.

Table 3: Composition of Vitamin Solution

Component	Stock solution	Quantity	Molar concentration in final medium
Thiamine HCl	-	200 mg	2.96×10^{-7} M
Biotin	1.0 g/L dH_2O	1ml	2.05×10^{-9} M
$CuSO_4 .5H_2O$	1.0 g/L dH_2O	1 ml	3.69×10^{-10} M

Primary stock solution was prepared using biotin and $CuSO_4.5H_2O$. The vitamin solution was prepared by dissolving thiamine and 1ml of the primary stocks to 950ml of distilled H_2O. The final volume was brought to 1litre with distilled H_2O. It was filter sterilized and stored in refrigerator.

Culture conditions

Experiments were performed in 250ml Erlenmeyer flasks with a volume of 100ml of f/2 media. 3ml of phytoplankton culture was inoculated in f/2 media supplemented with different concentrations of humic acid. The experimental set up was maintained as triplicates and the cultures flasks were periodically agitated. The most important parameters regulating phytoplankton growth such as light, pH, salinity and temperature were maintained at optimal condition.

Biomass estimation:

Phytoplankton growth was determined based on the cell density and optical density techniques. The cultures were recovered and the optical density (OD) value of the growth was assessed using Shimadzu UV/Vis 2600 Spectrophotometer at a wavelength range of 400–700nm [6]. The growth was analyzed in every alternate day and the biomass concentration (C_b, gL^{-1}) was calculated as follows: $C_b=0.38 \times OD_{625}$

Estimation of proximate composition:

Carbohydrate, protein and lipid content were quantified based on the methods of [7], [8] and [9], respectively.

Animal rearing and maintenance:

Healthy shrimp *Litopenaeus vannamei* (Zoea stage1- post larval stage1) were cultured in tanks with sterilized seawater filtered at 1µm, salinity of 37ppt and temperature of 27± 1°C under continuous aeration. Brood stock maintenance (from Nauplii to post larval stage 1) and experimental study was performed at Alwin aquatech shrimp hatchery, Marakkanam, Tamil Nadu.

Conditions of the experimental study:

i. The experimental cultures were fed with the following: (50% phytoplankton *Thalassiosira pseudonana* + 30% *artemia*, depends on the stage of animal).

ii. Phytoplankton were fed for 4 times/day on every 6hr interval (IST 6.00, 12.00, 18.00 and 24.00).

iii. Density of alga in the experimental setup: stocking culture (i.e. density is 25000 - 30000 cells/ml) × 4 times of each stage of the animal in the volume of each tank.

iv. Additional dietary supplement: Exorich (1ppm), Vitamins (1ppm) which includes nutritional components of feed including 40% protein, 9.5% fat, 3% fibre, 15% ash and 10% humidity.

Experimental analysis of the growth of larva in the hatchery:

Survival:

The formula for calculating the survival rate in each stage (Ts):

$$Ts\ (\%) = T_2/T_1 \times 100$$

in which: T_1 count is the number of larvae in the previous period; T_2 the numbers of larvae were counted at a later stage (pcs).

The formula for calculating the cumulative survival rate:

$$M\ (\%) = M_2/M_1 \times 100$$

in which: $M_1$the initial draft of larvae; M_2 count is the number of larvae at a later stage.

Length of the shrimp body:

30 larvae/ experimental tank were collected to test once.

Absolute length growth:

$$DG\ (mm/day) = Ls - Lt/\Delta t$$

Length relative growth:

$$GR\ (\%/day) = \ln(Ls) - \ln(Lt)\ /\Delta t \times 100$$

DG is the growth of the absolute length; SGR is the growth of the relative length; Ls is the index of average body length was measured at time t_2; Lt is the index of average body length was measured at time t_1; Δt is the period of time between two measurements.

3. RESULTS:

Fig. 1: Biomass of *Nannochloropsis* sp. with various concentrations of humic acid (A- 100µL, B- 200µL & C -300µL).

Control A B C

Fig. 2: Biomass of *Tetraselmis* sp. with various concentrations of humic acid (A- 100µL, B- 200µL & C -300µL).

Control A B C

Fig. 3: Biomass of *Dunaliella salina* with various concentrations of humic acid (A- 100µL, B- 200µL & C -300µL).

Control A B C

Fig. 4: Biomass of *Spirulina* sp. with various concentrations of humic acid (A- 100µL, B- 200µL & C -300µL).

Control A B C

Fig. 5: microscopic view of *Nannochloropsis* sp. at various concentrations of humic acid (A- 100µL, B- 200µL & C -300µL).

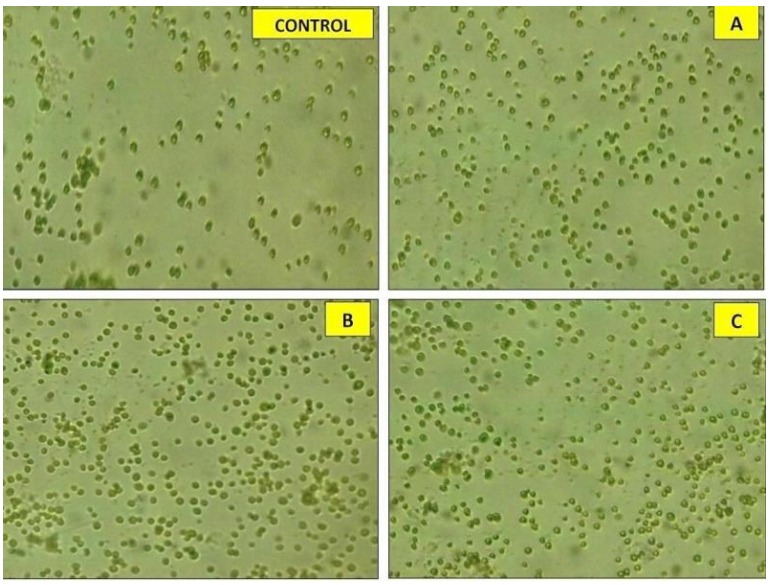


Fig. 6: microscopic view of *Tetraselmis sp.* at various concentrations of humic acid (A - 100µL, B - 200µL & C- 300µL).

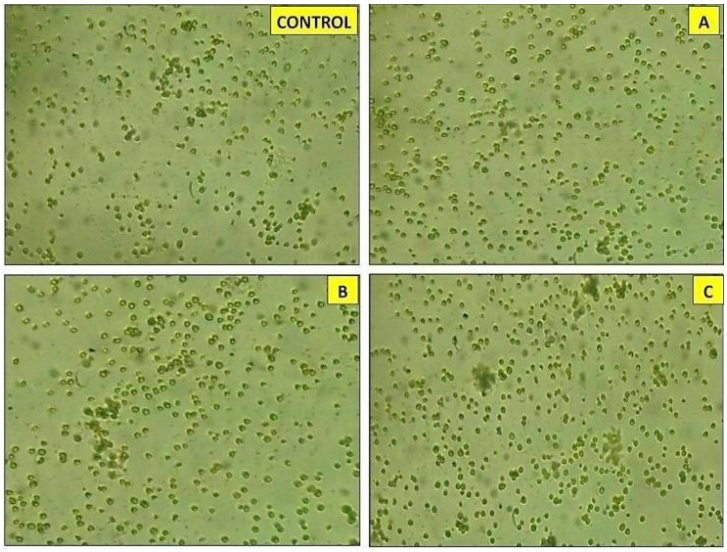
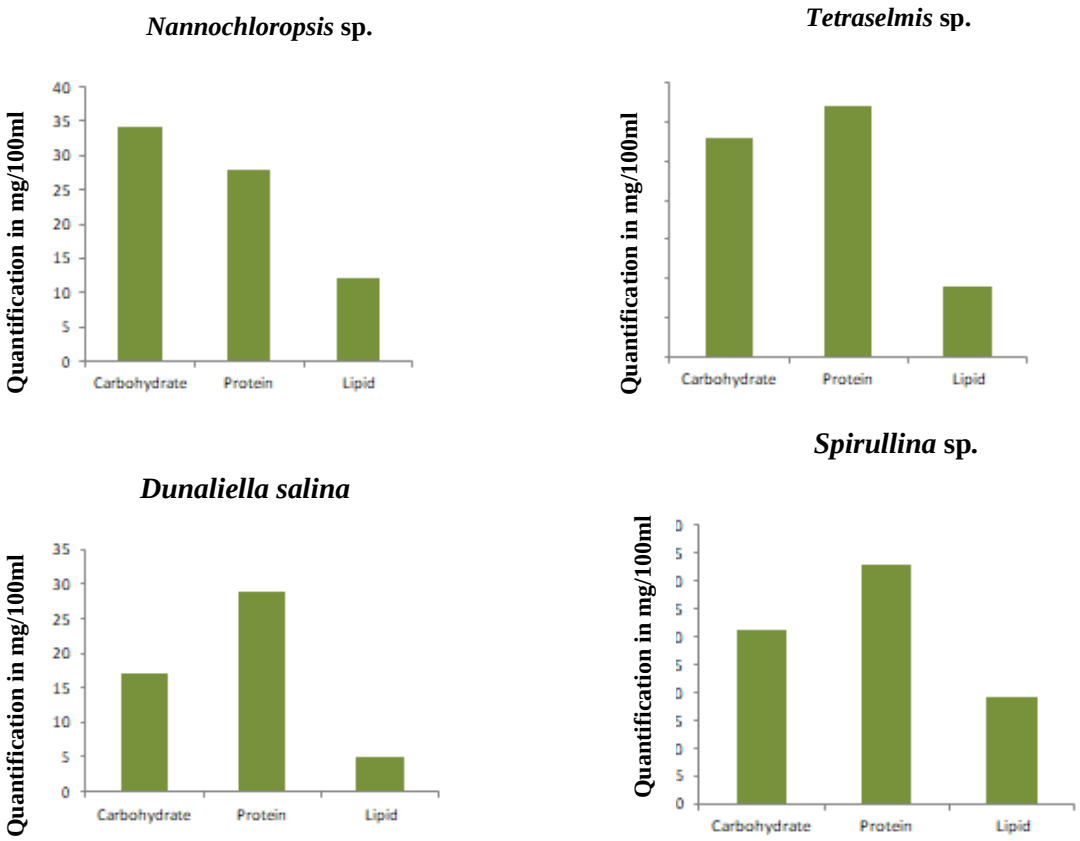


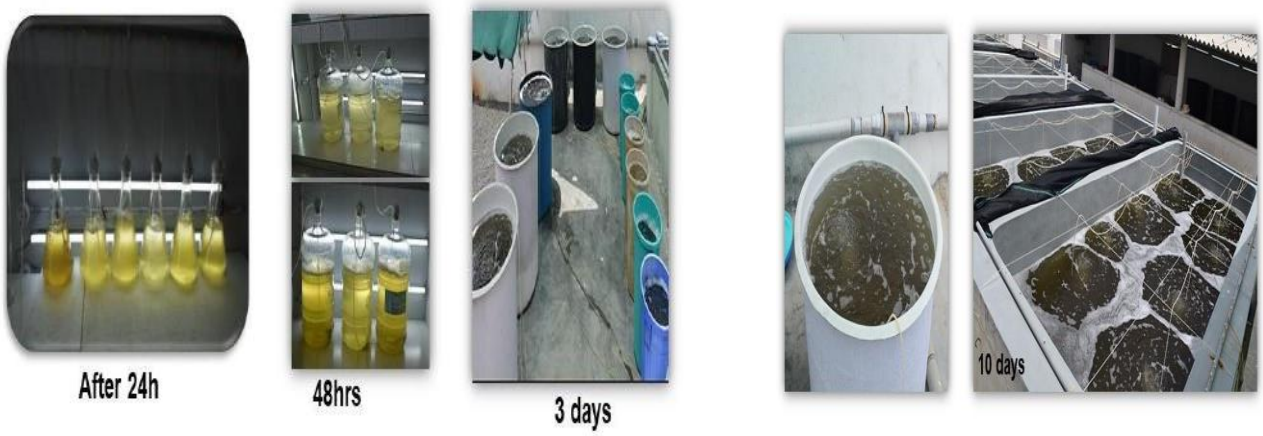
Fig. 7: Scaling up of phytoplankton cultures

Fig. 8: Proximate composition analysis



The biomass range of *Nannochloropsis* sp. was found to be increased when it is cultured in f/2 medium supplemented with 200µl of humic acid and it was also observed that the growth of phytoplankton declined when it is supplemented above the optimal concentration. The growth of *Tetraselmis* sp. was found promisingly increased when it is cultured in f/2 medium supplemented with humic acid (100µl - 300µl) concentration when compared to the control (f/2 medium without humic acid). The growth of phytoplankton had gradually increased as the concentration of humic acid increases in the medium. The growth of *Dunaliella salina* was not promising after treating with humic acid in the culture medium. *Spirulina* sp. increased in its biomass when cultured in medium supplemented with humic acid which gained importance and international demand for its high value phytonutrients. It has also generated much commercial interest in recent years, due to its economic value.

Fig. 9: Hatchery scale up of *Thalassiosira pseudonana*



The results obtained on the effect of both feeds on the white leg shrimp larvae at different stages: Normal – *Thalassiosira pseudonana* alone; Supplemented- HA aided *Thalassiosira pseudonana*

Table 4: Survival of various larval stages of *L. vannamei*

Larval stage	Survival rate (%)	
	Normal	Supplemented
Zoea 1- Zoea 2	82.10 ± 2.25	82.50 ± 2.45
Zoea 2 - Zoea 3	83.35 ± 1.40	83.20 ± 1.70
Zoea 3 - Mysis 1	84.65 ± 1.15	85.45 ± 1.35
Mysis 1- Mysis 2	85.85 ± 2.45	86.85 ± 2.75
Mysis 2 - Mysis 3	88.15 ± 1.85	88.25 ± 1.95
Mysis 3 - post larval stage 1	90.25 ± 1.25	91.45 ± 2.15

Table 5: Total length of the *L. vannamei* larvae at different stages

Stage	Total length (mm)	
	Normal	Supplemented
Zoea 1- Zoea 2	1.165 ± 0.035*	1.175 ± 0.045*
Zoea 2 - Zoea 3	1.275 ± 0.015*	1.290 ± 0.025*
Zoea 3 - Mysis 1	1.855 ± 0.040*	1.875 ± 0.050*
Mysis 1- Mysis 2	2.250 ± 0.035*	2.251 ± 0.035*
Mysis 2 - Mysis 3	2.880 ± 0.020*	2.885 ± 0.050*
Mysis 3 - post larval stage 1	3.530 ± 0.065*	3.540 ± 0.075*

*The data in the same column with different letters hat is significant difference *($p < 0.05$).*

Table 6: Absolute and relative growth rate to body length of *L. vannamei* larva

Stage	DG (mm/day)		SGR (%/day)	
	Normal	Supplemented	Normal	Supplemented
Zoea 1- Zoea 2	0.320 ± 0.007	0.350 ± 0.002	25.25 ± 0.025	25.75 ± 0.02

Zoea 2 - Zoea 3	0.940 ± 0.002	0.950 ± 0.004	35.40 ± 0.150	35.50 ± 0.150
Zoea 3 - Mysis 1	0.750 ± 0.015	0.760 ± 0.015	44.75 ± 0.045	45.75 ± 0.075
Mysis 1- Mysis 2	0.575 ± 0.025	0.580 ± 0.025	64.00 ± 0.055	65.00 ± 0.035
Mysis 2 - Mysis 3	1.755 ± 0.005	1.760 ± 0.015	85.00 ± 0.050	85.25 ± 0.050
Mysis 3 - post larval stage 1	1.820 ± 0.002	1.820 ± 0.005	88.25 ± 0.005	88.45 ± 0.145

The results in Table 4, 5, & 6 showed that, when using two form of feed, the survival rate of larvae is found be slightly different. In each larval stage of *L. vannamei*, i.e. Zoea 1- Post larval stage 1, the survival rate is found to be increased when compared with normal, the difference between them has the statistical significance ($p < 0.05$). According to the study, when stocking larvae in humic acid supplemented *Thalassiosira pseudonana, artemia* + feed in all stages impacted survival rate percent of 82.5% to 91.4%. The average growth at Zoea stage 1 to Zoea stage 2 was found to be 1.175 ± 0.045* (HA supplemented) where as in normal it was seen as 1.165 ± 0.035* in mm. The maximum growth of 3.540mm was observed at post larval stage 1 that are provided by HA supplemented phytoplankton. The results also demonstrated the increase in the relative growth rate from Zoea stage 1 to Zoea stage 2 and it was found to be ± 0.02 per day. Thus the present study illustrated that, when compared with normal feed formulation, the live feed supplemented with humic acid enhances the metabolism of experimented animal (*L.vannamei*) thereby, improving the rate of survival and growth.

4. DISCUSSION:

The supplementation of humic acid resulted in high productivity in phytoplankton over a short period of time and can be used as a potent constituent in phytoplankton feed formulation. Studies showed that humic acid has the ability to increase the productivity of phytoplankton in environment and laboratory experiments in which most of the reports evidenced marine phytoplankton growth [10]. [11] indicated that the biologically active and ingredients of humic substances stimulate growth of marine phytoplankton. The supplementation of humic acid favored the increase of proximate composition in *Nannochloropsis* sp., *Tetraselmis* sp., *Dunaliella salina* and *Spirulina* sp. which signifies its higher economic value [12]. [13] demonstrated that dinoflagellate responds to humic acid additions with an increase in growth rate and [14] reported that humic additions could stimulate the growth and nitrogen utilization of phytoplankton. The use of humic acid as culture media supplement will substantially reduce the cost of biomass production of phytoplankton, however, this needs elaborated research and optimization studies for commercial applications. Since, humic acid supplemented phytoplankton can be utilized as an effective natural feed for aquaculture enhancement, upgradation of microalgal technology from pilot scale to commercial level is beneficial by overcoming the associated challenges and limitations.

5. CONCLUSION

In the past few decades, tremendous advances have been made in the field of phytoplankton technologies for combating numerous techno-economic hurdles and improving biomass production. The major limitations with the use of phytoplankton as an alternative feedstock is the cost involved in its cultivation. The bottleneck of the phytoplankton culturing is the economic production of biomass due to the high recurring cost of nutrient media required for the cultivation. Large-scale cultivation of phytoplankton with the supplementation of humic acid will decisively contribute to the development of a sustainable industry for

biomass production as well as generating cost-effective high-value products. It is necessary to develop successful culturing technologies for targeted production of biomass to make the phytoplankton production sustainable, feasible and economically viable.

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