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Humus and BD500 as alternative culture media for biomass production of marine micro algae *Nannochloropsis* sp.

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INTRODUCTION

Microalgae are attractive for commercial exploitation because they grow fast by doubling their biomass quickly for which they require only minimal mineral nutrients[5].Moreover, microalgae are currently being considered as suitable raw material for production of biofuel[5] carotenoids and PUFAs[32]. However, economically valuable production of such compounds still depends on enhancing algal biology and bioprocess engineering aspects crucial for its massive production.

Nannochloropsis sp. which is the sole marine genus of the Eustigmatophyceae[14], is frequently used in commercial aquaculture due to its nutritional values such as sterols[43] polyunsaturated fatty acids[37] especially C20:5ω3 (EPA)[25] and pigments, such as zeaxanthin, canthaxanthin and astaxanthin[20]. Despite the widespread use of *Nannochloropsis* sp. as aquaculture feed, it's industrial exploitation in mass culture systems as a source of EPA cannot yet compete with fish oil due to high production costs[44]. These costs can only be reduced by implementation of daily culture control to optimize production, which involves in-depth knowledge of independent and combined interaction effects of several key factors upon culture growth and dynamics. For better growth of *Nannochloropsis* sp. carbon and nitrogen source should be in sufficient concentration. Carbon source can be from atmospheric CO_2 and for nitrogen supplement with different sources are available including- KNO_3, $NaNO_3$, $Ca(NO_3)_2 4H_2O$, urea and glycine are supplied and compared in that urea revealed better result than others for biomass increasing[37]. Pacheco-Ruiz et al[24] has recommended the use of organic fertilizer as a source of nutrients for algal production system. Various organic nonconventional media were tried for the culture of micro algae include agricultural fertilizers and soil extracts[13,19,21]. Valenzuela-Espinoza et al.[42] has prepared a low-cost media alternative to f/2 medium for cheaper production of micro algae.

In this study humic acid and biodynamic manure are used as organic fertilizer for algal culture. Humic acid is a matured product of organic manure after its complete microbial degradation has a complex mixture of C, H, N and O as main components in the form of mainly alkyl, aromatic, carboxyl, quinone and further complexed with other inorganic forms N, amionacids, proteins, sugars, micronutrients sometimes has plant growth hormones as functional groups in their negative charged free bonds, this binding shows its cation exchange capacity (CEC). This capacity qualifies any given humic acid as a good fertilizing agent to any living beings (cells). BD 500 is made from cow manure (fermented in a cow horn that is buried in the soil for six months through autumn and winter) and is used as a soil spray to stimulate root growth and humus formation[39]. In the present study, humic acid and biodynamic manure were investigated for the growth of the alga

Nannochloropsis sp.

MATERIALS AND METHODS

Algal culture

Nannochloropsis sp. used in the present study was obtained from the culture collection of Shri AMM Murugappa Chettiar Research Centre (MCRC), Chennai. Culture was maintained by growing in 5L conical flasks containing 3L of sterile f/2 culture medium[12]. Algal cultures were established by inoculating 100 ml of a culture (2×10^6 cells ml^{-1}) growing in exponential phase. These were maintained in unialgal condition in the laboratory and were kept under sufficient light (2000 lux) and temperature (26-28°C) conditions with a pH of 8.2±1 (Guillard 1975).

Preparation of humic acid and biodynamic manure

Humic acids (A,B&C) and Biodynamic manure (BD500) were obtained from MCRC. The humic acids used for this study are from different sources and extraction by following different methods. Water extract (WE) of BD500 was prepared by adding 100 ml of distilled water to 20 g of BD500 and mixed well then it was filtered by filter paper to collect the water extract.

Experiment 1

The culture media was prepared in artificial sea water using the procedure of Provasoli et al., 1957. Humic acids were added at different concentrations (0.1-0.5%)[27].

Experiment 2

To the prepared artificial sea water 0.1% humic acid was supplemented with 0.001% vitamin (B_{12}&B_1).

Experiment 3

0.1% humic acid was supplemented with 6% biodynamic manure

Algal cultures were established by 10% inoculum (2×10^6 cells ml^{-1}) growing in exponential phase. These were maintained in unialgal condition in the laboratory and were kept under sufficient light (2000 lux) and temperature (26-28°C) conditions with a pH of 8.2±1[11]. After 20 days of growth the cells were counted.

All formulations were prepared for 100 ml and maintained in 250 ml conical flasks in triplicates and *Nannochloropsis* sp. (10% inoculum contain 2×10^6 cells ml^{-1}) was inoculated.

Analysis of Physico-chemical properties in humic acid and biodynamic manure

pH and EC[3], Total nitrogen (N)[4], phosphorus (P)[22], potassium (K)[18], calcium magnesium sodium and micro nutrients (Iron, Zinc, Manganese and Copper)[41] were analyzed using the standard procedure.

Growth Measurements

The growth of the microalgal cultures was measured by direct counting of the cells using a haemocytometer (Neubauer). The mean value of three counts was taken for each sample.

Statistical analysis

Each measurement was done in triplicate and the mean and standard deviation of the experimental results was calculated using SPSS 14.0. Where differences in ANOVA tests for treatment were significant at the 0.05 level, means were separated using Turkey test values.

RESULTS AND DISCUSSION

The physico chemical parameter of humic acid prepared from different sources (A, B&C) and biodynamic manure are presented in TABLE 1. The nutrients required for the majority of microalgae include Nitrogen (N) (> 0.2 µmol N L^{-1}), Phosphorus (P) (<0.2 µmol P L^{-1}) and Potassium (K) at low concentrations ensure that agricultural fertilizers as suitable substitute for commercial algal production[35,38] According to Reddfield[36] N:P ratio of 7:1 is the range of balanced nutrient for culturing algae. Higher ratios result in P limitations and a reduction in algal growth[6].

Nannochloropsis sp. was cultured in different concentration from 0.1-0.5% of humic acid. The initial concentration of 0.1% humic acid has shown $4x10^6$ cells ml^{-1} maximum growth on 7th day when compared with the culture grown in f/2 medium of $7x10^6$ cells ml^{-1}(Figure. 1). The study has proved clearly that as concentration of humic increases the algal growth decreases. This indicates that very less concentration of humic acid is necessary for algal growth. Our result is in accordance to Droop[8] who has found that the growth of *S. costutum* doubled when the “humic” fraction of soil humus was added to the synthetic medium. Prakash and Rashid[27] has demonstrated a stimulatory action of small amounts of humic substances isolated from river water, soil, and marine sediments on the growth of marine dinoflagellates. Previous reports also suggest that the terrigenous humic material through land drainage resulted in biological conditioning of coastal waters in favor of high phytoplankton production[26,28]. Humic substances regardless of their origin seem to have considerable metal-complexing capacity; their stimulating effect on marine phytoplankton may be largely a chelation response[26].

In the present study 0.001% vitamin (B_{12}&B_1) supplemented to the different sources of humic acid has shown a marginal increase in the cell count in the range of $4x10^6$ cells ml^{-1} Whereas with the supplementation of 6% biodynamic manure there was dramatic increase in the cell count to $7\text{-}8x10^6$ cells ml^{-1}(Figure. 2). This result agrees with earlier works reporting that the biomass production can be affected

TABLE 1 : Physicochemical properties of Humic acids and biodynamic manure (BD500)

Sl.No	pH	EC	N	P	K	Ca
		µS/cm	(%)	(%)	(%)	(%)
HA1	7.65 ± 0.16	1537.83 ± 17.22	0.13 ± 0.001	0.07 ± 0.001	0.41 ± 0.012	0.015 ± 0.001
HA2	8.01 ± 0.19	1208.58 ± 10.51	0.19 ± 0.002	0.12 ± 0.008	0.07 ± 0.002	0.011 ± 0.001
HA3	7.89 ± 0.14	1463.92 ± 69.10	0.05 ± 0.002	0.14 ± 0.002	0.14 ± 0.002	0.04 ± 0.001
BD500	7.32 ± 0.23	2343.87 ± 49.69	1.12 ± 0.024	1.12 ± 0.035	0.26 ± 0.008	0.98 ± 0.031
Sl.No	**Mg**	**Na**	**Fe**	**Mn**	**Zn**	**Cu**
	(%)	(%)	(ppm)	(ppm)	(ppm)	(ppm)
HA1	0.002 ± 0.000	0.03 ± 0.000	86 ± 4.145	1.8 ± 0.087	0.8 ± 0.009	11.4 ± 0.549
HA2	0.009 ± 0.001	0.15 ± 0.001	349 ± 29.386	1.6 ± 0.135	2.6 ± 0.023	9.4 ± 0.791
HA3	0.013 ± 0.000	0.02 ± 0.001	111 ± 1.909	0.8 ± 0.014	1 ± 0.047	2 ± 0.034
BD500	0.82 ± 0.026	0.22 ± 0.005	2187 ± 76.982	413 ± 12.886	57 ± 1.208	2 ± 0.062

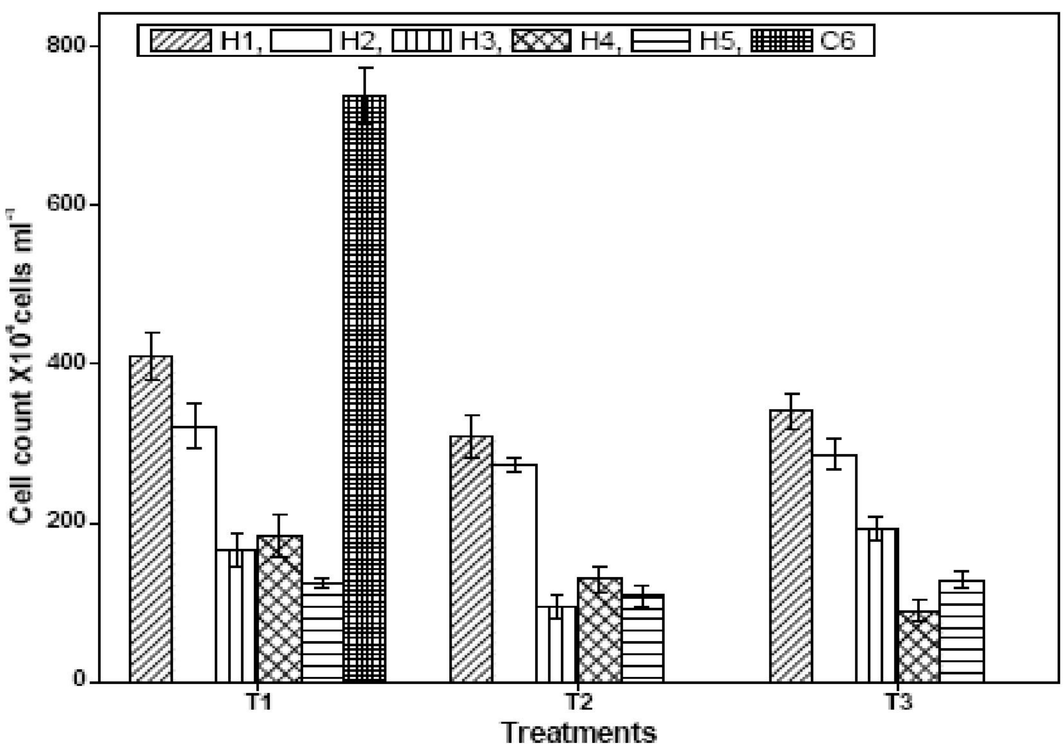

Figure 1: Effect of different concentration of Humic acids on algal cell growth H1-100mg of humic acid+ASW, H2-200mg of humic acid+ASW H3- 300mgof humic acid+ASW, H4-400mgof humic acid+ASW, H5-500mgof humic acid+ASW, C6-F/2 Medium, T1-Humic acid A, T2- Humic acid B, T3- Humic acid C

by the concentration of nutrients but not by the compound utilized[9,13]. The N and P contribution from humic compounds also has limited effect, since increasing N and P beyond a certain concentration in the medium did not proportionately increase the growth of the *Nannochloropsis*. On the contrary, while at high

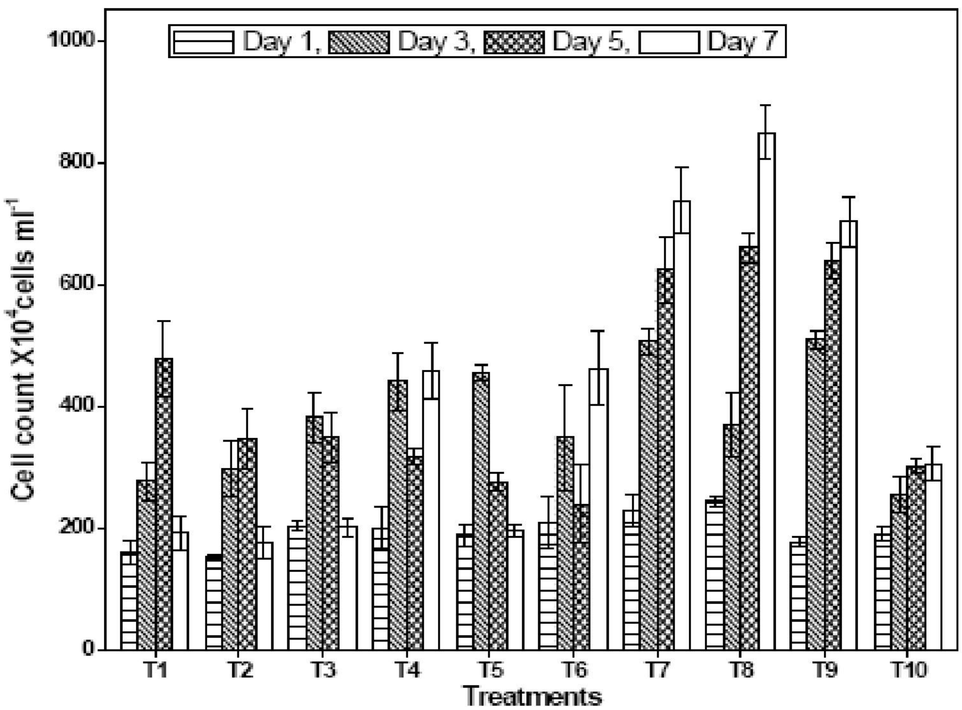

Figure 2 : Impact of humic acid and BD500 on growth of *nannochloropsis sp.* T1-HUMIC ACID A+ASW, T2 –HUMIC ACID B+ASW, T3- HUMIC ACID C+ASW, T4- HUMIC ACID A +Vitamin+ASW, T5 - HUMIC ACID B +Vitamin+ASW, T6-HUMIC ACID C +Vitamin+ASW, T7 HUMIC ACID A+BD500+ASW, T8- HUMIC ACID B+BD500+ASW, T9- HUMIC ACID C+BD500+ASW, T10 -ASW

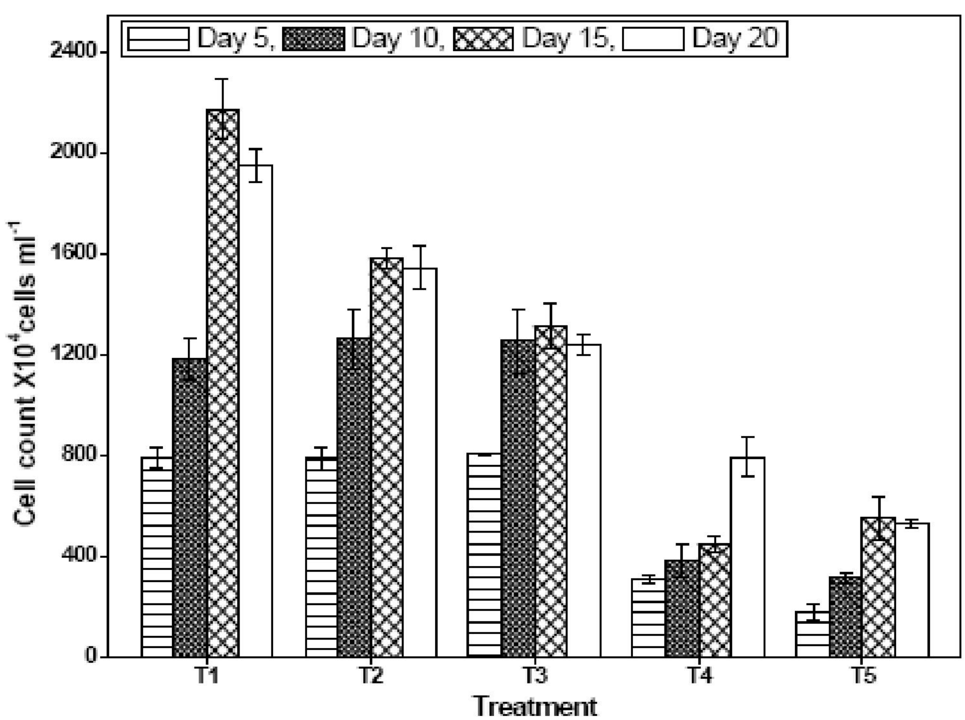

Figure 3 : Impact of humic acid and BD500 on growth of *nannochloropsis sp.*T1- HUMIC ACID A+BD500+ASW, T2- HUMIC ACID A +BD500+ASW, T3- HUMIC ACID C +BD500+ASW, T4- BD 500+ASW, T5- ASW

concentrations of N and P, both the growth rate and the total yield decreased, our results suggest that humic acid is capable of removing the negative effect of high dosage of N and P. Nitrogen and phosphorus supply have a direct influence on the growth of microalgae[23]. Carbon, nitrogen and phosphorus are the nutrient elements required in greatest quantity by algae. Carbon and nitrogen metabolism are tightly linked[17]. High CO_2 enhances the nitrogen assimilation of microalgal cells[15], and in turn, nitrogen supply can impact upon the utilization of inorganic carbon by microalgae[10].

When 6% of biodynamic manure was supplemented to the humic acids obtained from different sources the humic acid A has shown a significant growth of $2x10^7$ cells ml^{-1} compared to the humic substance obtained from other sources (Figure. 3). According to Huerlimann et al.[16], when grown in f/2 medium, *Nannochloropsis* sp. showed a growth of ~2.5 x 10^7 cells ml^{-1}. Abu Rezq et al.[1] has reported maximum cell density of *Nannochloropsis* sp. as ~32 x 10^6 cells ml^{-1}. One of the best parameters to monitor microalgae production is the estimation of growth over a certain period of time[2]. In lab grown cultures, microalgae biomass reaches a peak level after 25th day and then starts decreasing[7]. The apparently low molecular weight humic fractions have a high total acidity and a high number of carboxyl groups associated with their metal holding capacity. According to Rashid[34] the lowest molecular weight fraction complexed 2 to 6 times more metals than any other and the metal complexing ability decreased with increasing molecular weight; this could explain why the apparently low molecular weight HA fractions were the most active in growth stimulation.

Role of humic matter in the development and growth of terrestrial plants has been fairly studied extensively whereas; studies on marine algae are almost nonexistent. Humic substances may have some stimulating effect on the phytoplankton production in the sea has been suggested by a number of authors[40], but there is little direct evidence available. The biologically active ingredients of humic matter that stimulate growth of marine phytoplankton are perhaps as varied as they are complex and offer a fertile field for future investigation.

REFERENCES

[1] T.S.Abu-Rezq, L.Al-Musallam, J.Al-Shimmari, P.Dias; Hydrobiologia., **403**, 97-107 **(1999)**.

[2] E.W.Becker; Microalgae: biotechnology and microbiology (in press).

[3] M.P.Bernal, A.F.Navarro, M.A.Sanchez-Monedero, A.Roig, J.Cegarra; Soil Biol.Biochem., **30**, 305-313 **(1998)**.

[4] J.M.Bremner; Total nitrogen, 1149-1178, in C.A. Black, Couvering Eds. Methods of Soil Analysis, Part 2, American Society of Agronomy, Madison **(1965)**.

[5] Y.Chisti; Biotechnology Advances, **25**, 294-306 **(2007)**.

[6] D.L.Correll; Journal of Environmental Quality, **27**, 261-266 **(1998)**.

[7] C.Dayananda, R.Sarada, M.Usha Rani, T.R.Shyamala, G.A.Ravishankar; Biomass Bioenergy, **31**, 87-93 **(2007)**.

[8] M.R.Droop; Comments (Proc. Int. Interdisciplinary Conf., 2nd), in C. H. Oppenheimer, Couvering Edition. 'Marine biology' v. 2, N.Y. Acad.Sci.,158-159 **(1966)**.

[9] J.Fabregas, C.Herrero, J.Abalde, B.Cabezas; Aquaculture, **50**, 1-11 **(1985)**.

[10] M.Giordano, J.Beardall, J.A.Raven; Annu.Rev. Plant Biol., **56**, 99-131 **(2005)**.

[11] R.R.L.Guillard; Culture of phytoplankton for feeding marine invertebrates, in W.L.Smith, M.H.Chanley, Couvering Edition. Culture of Marine Invertebrate Animals, Plenum Press, New York (USA), 26-60 **(1975)**.

[12] R.R.L.Guillard, J.H.Rhyter; Grans.Canadian Journal of Microbiology, **8**, 229-239 **(1962)**.

[13] C.Herrero, A.Cid, J.Fabregas, J.Abalde; Aquacult Eng., **10**, 99-110 **(1991)**.

[14] D.J.Hibberd; Botanical Journal of the Linnean Society, **82(2)**, 93-119 **(1981)**.

[15] H.Hu, X.Zhang; World J Microbiol Biotechnol, **24**, 891-894 **(2008)**.

[16] R.Huerlimann, R.de Nys, K.Heimann; Bio technology and Bioengineering, **107**, 245-257 **(2010)**.

[17] H.C.Huppe, D.H.Turpin; Annu.Rev.Plant Physiol. Plant Mol.Biol., **45**, 577-607 **(1994)**.

[18] M.L.Jackson; Soil chemical analysis **(1973)**.

[19] J.L.Lopez-Ruýz, R.Garcia-Garcia, M.S.FerreiroAlmeda; Aquacult.Eng., **14**, 367-372 **(1995)**.

[20] L.M.Lubian, O.Montero, I.Moreno-Garrido, E.Huertas, C.Sobrino, M.Gonzalez-Delvalle, G.Pares; J.Appl.Phycol., **12**, 249-255 **(2000)**.
[21] M.Nieves, D.Voltolina, M.T.Sapien, H.Gerhardus, A.I.Robles, M.A.Villa; Revista Italiana di Acquacolture, **31**, 81-84.
[22] S.R.Olsen, C.V.Cole, F.S.Watanabe, A.L.Dean; Estimation of available phosphorous in soils by extraction with sodium bicarbonate (USDA) Circular number, 939 **(1954)**.
[23] O.V.H.Owens, W.E.Esaias; Annu.Rev.Plant Physiol., **27**, 461-483 **(1976)**.
[24] I.Pacheco-Ruiz, J.A.Zeertuche-Gonzalez, E.Arroyo-Ortega, E.Valenzuela-Espinoza; Aquaculture, **240**, 201-209 **(2004)**.
[25] V.Patil, T.Kallqvist, E.Olsen, G.Vogt, H.R.Gislerod; Aquaculture International, **15**, 1-9 **(2007)**.
[26] A.Prakash; Terrigenous organic matter and coastal phytoplankton fertility, in J. D. Costlow, Couvering Edition. 'Fertility of the sea', v. 2. Gordon and Breach 351- 368 **(1971)**.
[27] A.Prakash, M.A.Rashid; Dinoflagellates. Limnol. Oceanogr., **13**, 598-606 **(1968)**.
[28] A.Prakash, M.A.Rashid; The influence of hurniubzs on coastal phytoplankton productivity, in A.Ayala-Castanares, F.B.Phleger, Couvering Edition. Coastal lagoons, a symposium.Mem. Symp.Int.Lagunas Costeras, UNAM-UNESCO 431-438 **(1969)**.
[29] S.Prat, M.Smidova and A.L.Cincerova; Penetration and effect of humus substances (fractions) on plant cells.Int.Congr.Biochem.(5th) Abstr. Commun.Moscow, 329 **(1961)**.
[30] L.Provasoli; Organic regulation of phytoplankton fertility, in M. N. Hill, Couvering Edition. The sea, v. 2, Interscience publisher Inc., New York, 165-219 **(1965)**.
[31] L.Provasoli, J.J.A.Mclaughlin, M.R.Droop; Arch.Microbiol., **25**, 392-428 **(1957)**.
[32] O.Pulz, W.Gross; Applied Microbiology and Biotechnology, **65**, 635-648 **(2004)**.
[34] M.A.Rashid; Soil Sci., **111**, 298-306 **(1971)**.
[35] M.Rasmussen; Primary biomass production from marine algae, University of Aarhus: National Environmental Research Institute, Memorandum for VE-net, **(2007)**.
[36] A.C.Redfield; American Scientist, **46**, 205-221 **(1958)**.
[37] J.M.Rocha, J.E.Gracia, M.H.Henriques; Biomol.Eng., **20(4-6)**, 237-242 **(2003)**.
[38] SEI, A reviewof the potential ofmarine algae as a source of biofuel in Ireland **(2009)**.
[39] Steve Diver (1999). Biodynamic Farming and Compost Preparation, Alternative Farming Systems Guide. ATTRA – National Sustainable Agriculture Information Service, http://attra.ncat.org/attra-pub/biodynamic.html#preparations. Accessed May 26, **(2006)**.
[40] J.D.Strickland II; Production of organic matter in the primary stages of themarine food chain Strickland, in J.P.Riley, G.Skirrow, Couvering Edition. Chemical oceanography, v.1, Academic, New York 477-610 **(1965)**.
[41] H.L.S.Tandon; Methods of Analysis of Soils, Plants, Water and Fertilizers, Fertilizers Development and Consultation Organisation, New Delhi, India:144 **(1993)**.
[42] E.Valenzuela-Espinoza, R.Millán Núnez, F.Núnez Cebrero; Aquaculture Engineering, **25**, 207-216 **(2002)**.
[43] B.Veron, J.C.Dauguet, C.Billard; J.Phycol., **34**, 273-279 **(1998)**.
[44] C.W.Zhang, O.Zmora, R.Kopel, A.Richmond; Aquaculture, **195**, 35-49 **(2001)**.