Growth responses of *Chlorella* sp. to some selected variants of culture medium and in effluents of a Brewery

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ARTICLE INFO

Keywords:
Chlorella sp.
Microalgae
pH
CO_2
Brewery
Effluent

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ABSTRACT

Global warming poses a major threat to our civilization that has led to unsustainable development worldwide. Though there are different strategies for global warming mitigation, carbon sequestration in water column via microalgae is the eco-friendly, cost effective and sustainable tool to capture and utilize carbon in a beneficial way. Microalgae, *Chlorella* sp. was cultured in *ex situ* using (a) standard basal medium as control, (b) culture medium aerated, (c) exogenously introduced liquid carbon dioxide into the culture medium. The growth of *Chlorella* was also examined under different pH (4 - 11) of the culture medium as well as in various dilutions (0, 25, 50%) of effluents of a Brewery under autoclaved and non- autoclaved conditions. There was no marked difference in growth of *Chlorella* sp. between 0 - 166 hours and between 218 - 272 hours either, but significant difference ($P < 0.05$) in growth was clearly discernable during the peak period among the treatments. Exogenously introduced CO_2 or aeration of the culture medium did not significantly improve the growth over basal medium suggesting that optimal conditions of carbon or oxygen have prevailed in the basal medium of culture. There was a sharp dichotomy of pH effects on the growth of Chlorella as growth was distinctly higher at pH 8 to 11 compared to the remaining pH (4 - 6) showing no significant differences ($P > 0.05$) among themselves. *Chlorella* sp. grown in brewery effluent showed the maximum growth in 50% and 25% dilution of non autoclaved and autoclaved effluent of beverage factory respectively suggesting that autoclaving saved 25% of bacteria driven nutrient demand.

1. Introduction

Global warming poses major threat to our civilization that has led to unsustainable development worldwide. The primary reasons have been traced to the increasing trends of emission of greenhouse gases (GHGs) from industrial and anthropogenic sources into the atmosphere. These GHGs cause depletion of ozone layer thatprotects the atmosphere against UV radiation, thereby warming the atmosphere. As a result, the average concentration of CO_2 increased from 315 ppm in 1960 to 380 ppm in 2007 (IPCC, 2007).Further, there has been more than 35% increase in CO_2 emission worldwide since 1990.This concern has become the main stimulus for much research being carried out worldwide. Though there are different major strategies such as trapping of atmospheric $CO_2$from the atmosphere, long term oceanic storage and biofuel or biochar production, sequestration in water column via microalgae and macrophytes and carbon sink in bottom sediments of wetlands have been identified as a promising tool for mitigation of global warming. The acquisition of CO_2 through the biological process is eco-friendly, cost effective and sustainable to capture and utilize carbon in a profitable manner (Jana et al., 2016). It has several advantages for creation of mild conditions for CO_2 fixation and trapped $CO_2$is incorporated into carbohydrates and lipids and algal biomass can be transformed into biofuel and biochar (Peterhansel et al., 2010; Solovchenko and Goldber, 2013; Nogia et al., 2016) or natural algal food for fish and human consumption. This is a win-win strategy towards carbon mitigation and production of low carbon foot print fish, especially in tropical countries.

All photoautotrophs have to produce their own food through photosynthesis though their several modifications with xanthophylls and caretenoids occurs in higher plants (Eonseon et al., 2003).Microalgae are mostly photosynthetic and have chlorophyll embedded in the chloroplasts of microalgae(Hosikian et al, 2010).

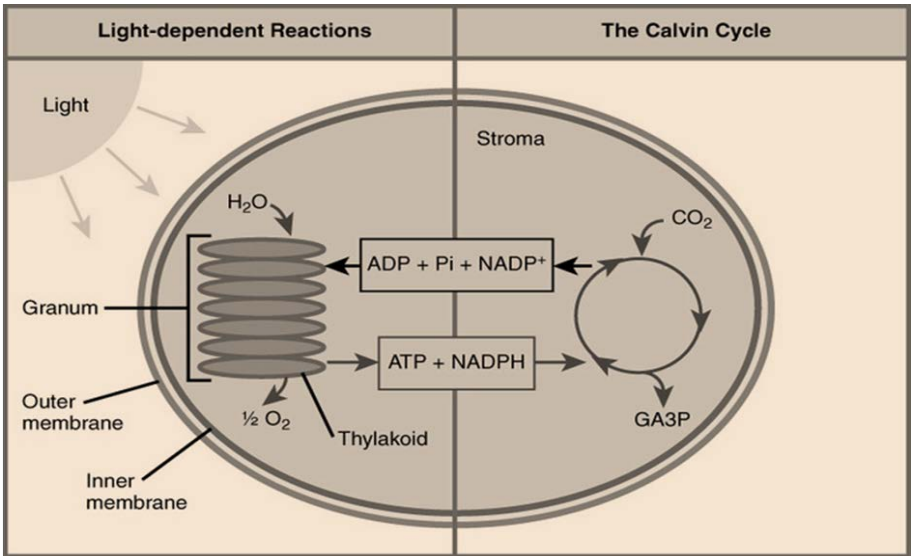
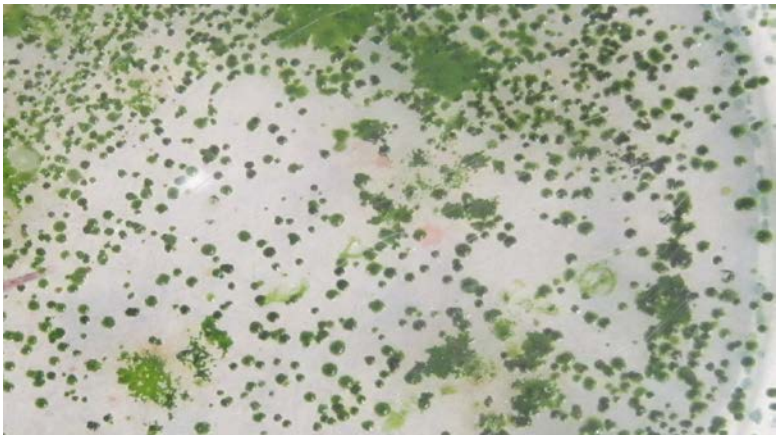


Fig. 1 A schematic representation of the photosynthetic process in microalgae

Photosynthesis is a process for the conversion of radiant energy into chemical energy with the help of $CO_2$and water. Altogethersix kinds of photosynthetic pigments (chlorophyll a, b, c, d, e and f) and many accessory pigments such as blue, red, brown, and gold are present in photosynthetic plant and algae. Microalgae are unique example where all kinds of photosynthetic pigments including chlorophyll e are present. Chlorophyll a is most essential to capture light wavelengths in the spectrum of sunlight. Chlorophyll b augments the ability of chlorophyll a to capture more sunlight. Chlorophyll c occurs mostly in dinoflagellates which also helps chlorophyll a to collect more sunlight, but it does not participate in photosynthesis beyond the initial stage. Chlorophyll d is found in marine red algae and cyanobacteria, and absorbs the infrared light of the electromagnetic spectrum. Chlorophyll e is a rare type found in some golden algae also assists in light capture (Pareek et al., 2017).Recently discovered chlorophyll f found in cyanobacteria and oxygenic bacteria absorbs further in the red (infrared light) than other chlorophylls. Chlorophyll e is a rare type found in some golden algae (Schliep et al., 2013).All types of chlorophyll viz. Chlorophyll a, b, c1, c2, c3, phytollated Chl c, d and f contains Mg. The molecular formula of chlorophyll d is $C_{54}H_{70}O_6N_4Mg$.

Photosynthesis is the conversion of photon energy into chemical energy to form glucose and water and oxygen is released (Fig. 1) as the byproduct. Chlorophylls are made up of lipid-soluble hydrocarbon tail ($C_{20}H_{39}$), a hydrophilic head and a magnesium ion at the centre. Microalgal photosynthesis occurs in two stages: the first stage is the photoactivation of chlorophyll when light energy is initially converted to chemical energy in the form of two compounds: NADPH, a source of energized electrons; and ATP, the versatile energy currency. The second stage is called Calvin cycle where sugar is produced (Fig. 1).The Calvin cycle starts with the capture of CO_2or carbon fixation from air into the organic molecule in the chloroplast (Raines, 2003).In this cycle when fixed carbon is reduced to carbohydrate (CH_2O) by the addition of electron provided by NADPH and ATP (Johnson, 2016) which are produced by light reactions of photosynthesis. In essence, both the steps are coordinated with each other because none of the steps require light directly.

Fig. 2 Culture of *Chlorella* sp. in agar medium



Chlorella is a freshwater single-celled green alga spherical in shape, about 2 to 10 µm in diameter, and is without flagella (Fig. 2). It contains the green photosynthetic pigments-chlorophyll a and b in its chloroplast. *Chlorella* is rich in protein and a good source of several B- complex vitamins and iron, minerals, antioxidants including vitamin C and carotenoids. *Chlorella* is available in a variety of dietary supplement and detoxification regimen in the form of liquid extracts, tablets and powders. It has immense medicinal properties to improve cholesterol and blood sugar levels in human being. Further, it is reported that chlorophyll may bind with specific cancer-causing agents to form molecular complexes and may be useful in blocking the carcinogenic effects of these agents (Solymosi and Mysliwa-Kurdziel, 2017).

With the help of carbon dioxide, water, sunlight, and a small amount of minerals, *Chlorella* multiplies rapidly in water. Because of this unique property, *Chlorella* has often been selected as carbon sequester (Cheng et al., 2006; Chiu et al., 2009; de Morais and Costa, 2007) at different temperatures and for cleaning the effluents of several industries. During outdoor cultivation with solar as the light source, biomass productivity is strongly affected by environmental factors such as irradiation, temperature (Ugwu et al., 2007), CO_2% and pH of the medium.

Despite considerable interest for *Chlorella* for as dietary supplement, medicinal properties, and mitigation options for global warming, no systematic studies have been focused on its mass production as influenced by different factors like pH, CO_2, nutrient level so as to harvest the beneficial option for different uses. The purpose of the present study is to examine the growth responses of *Chlorella* sp. in different pH of the medium under aerated and controlled conditions.

2. Materials and methods

Growth responses of *Chlorella* were examined in the laboratory using (a) standard basal medium as control, (b) culture medium aerated, (c) exogenously introduced liquid carbon dioxide into the culture medium (Fig. 3). *Chlorella* used in the study was procured from the stock culture maintained in the laboratory of ICEE, Kalyani University and grown in the basal algal culture medium(Hi Media) having the basal requirements (Table 1). All the methods for preparation of media, inoculation of *Chlorella* sp. were done following standard procedures of autoclaving of the media, serial dilution of the stock culture of *Chlorella* sp., in the range of 10^{5-6} andculture in 500 ml conical flasks in triplicate under artificial light conditions for 24 hours. The counts of *Chlorella* grown in different dilutions were enumerated in a microscope at different time intervals. The growth of *Chlorella* was also recorded in terms of density of culture medium determined in a Spectrophotometer at 680 nm against control.

Table 1. Composition of Bold Basal media.

Constituents	**gm/ L**
$NaNO_3$	25.0
$MgSO_4.7H_2O$	7.5
NaCl	2.5
K_2HPO_4	7.5
KH_2PO_4	17.5
$CaCl_2.2H_2O$	2.5
$ZnSO_4.7H_2O$	8.82 g
$MnCl_2.4H_2O$	1.44 g
MoO_3	0.71 g
$CuSO_4.5H_2O$	1.57 g
$Co(NO_3)_2.6H_2O$	0.49 g
H_3BO_3	11.42 g
EDTA	50.0 g
KOH	31.0 g
$FeSO_4.7H_2O$	4.98 g
H_2SO_4 (conc)	1.0 ml

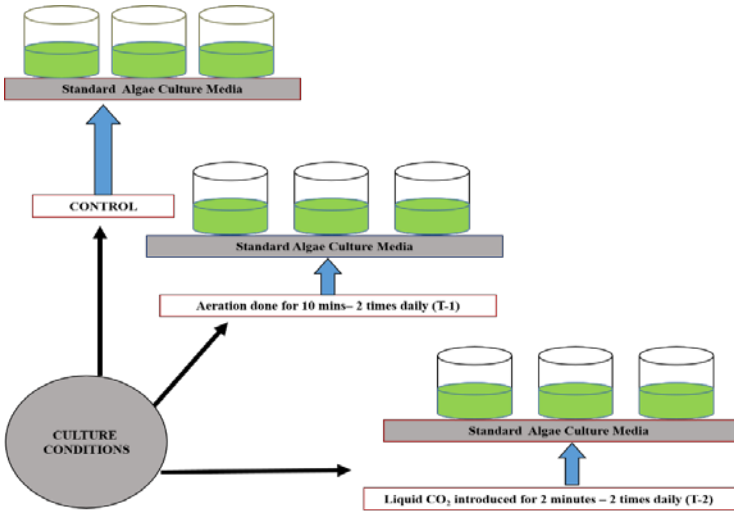


Fig. 3 Experimental design followed for growth of C*hlorella* sp. in algae culture media under aerated, exogenously introduced liquid carbon dioxide conditions

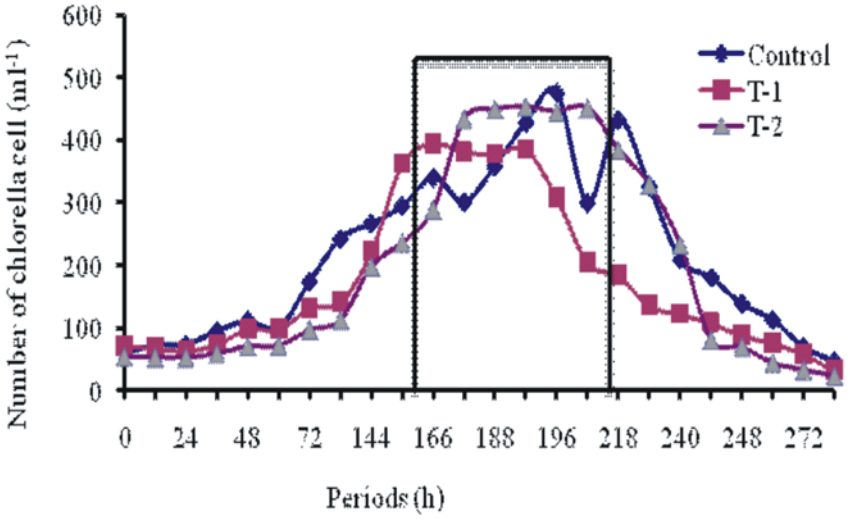


Fig. 4 Growth of *Chlorella* sp. under aerated, exogenously introduced liquid carbon dioxide conditions in algae culture media

2.1. Effect of pH of the culture medium

The effect of different pH of the culture medium was also determined; initial pH of the medium was measured with a digital pH meter. Different pH of the culture medium (4-11)was adjusted using 1 N HCl and N/44 NaOH. The culture was maintained in500 ml conical flasks. The growth of *Chlorella* sp. and the change of pH in the medium were recorded every 24 hours and adjustment of pH, if necessary, was done accordingly.

2.2. Growth of *Chlorella* in the effluents of beverage factory

In situ growth performance of *Chlorella* sp. was determined using different dilutions of the effluents of a Brewery factory. Effluent of beverage factory was collected and half portion of it was autoclaved under 15 lb pressures at 121°C for 20 minutes and then cooled to the ambient temperature where half part was not autoclaved as served as control. Both autoclaved and non autoclaved effluents werediluted by25% and 50% in conical flasks and volume was made up to 500 ml. Dilution was made by adding sterile water where one set of wastewater was kept as raw effluent. Culture of *Chlorella* was then inoculated in different dilutions of brewery effluents and allowed to grow under artificial light conditions in the laboratory. Growth of the algae species was measured at 680 nm in spectrophotometer (Shimadzu UV-visible Spectrophotometer, Model UV-1800) after every 24 hours from the culture till the growth cycle of *Chlorella* terminated.

3. Results and discussion

3.1. Growth of *Chlorella* sp.

There was no marked difference in growth of *Chlorella* sp. between 0 - 166 hours and between 218 - 272 hours either, but significant difference ($P<0.05$) in growth was clearly discernable during the peak period of growth ranging from 166 to 218 hours (Fig. 4). This was followed by a declining phase in all the three conditions including the control. The results of the study showed that exogenously introduced CO_2 or aeration of the culture medium did not significantly improve the growth over basal medium suggesting that optimal conditions of carbon or oxygen have prevailed in the basal medium of culture.

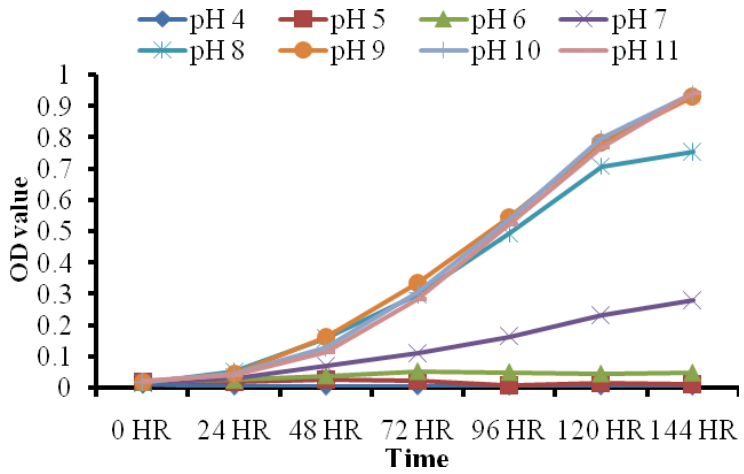


Fig. 5 Growth of *Chlorella* in different pH of culture media

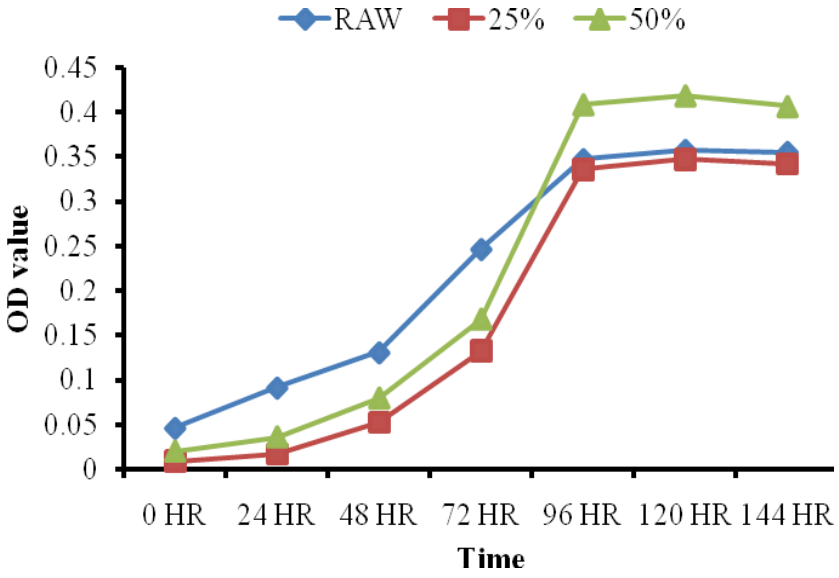


Fig. 6 Growth of *Chlorella* in non autoclaved effluent of Beverage factory

3.2. Growth of *Chlorella sp.* at different pH

There was a strong dichotomy of pH effects on the growth of *Chlorella*. There were clear-cut effects of pH of the culture medium on the growth of Chlorella sp. as growth was higher at pH 9 to 11 compared to the remaining pH showing no significant differences (P>0.05) among themselves. There was no difference during the lag phase of growth, but the differences tended to become more pronounced gradually after 48 hours when a strong dichotomy in growth was registered. The growth under pH of 8 - 11 remained distinctly higher than the rest of the pH of the medium tested. The growth at pH 7.0 remained higher than pH 4.0 - 6.0 (Fig. 5).

Optimal pH for growth of several algal species is reported to be in the range 7.5-8.5 (Acién Fernández et al., 2001; Chisti, 2008; Marcel et al., 2003; Molina et al., 2001), although there are species that can also grow at much higher pH (Ogbonna et al., 2000). pH values of beyond 10 can easily be reached in algal cultures in the absence of significant buffer or CO_2 supply and can lead to inhibition of growth (Qiang et al., 1996; Richmond and Cheng-Wu, 2001; Zhang and Richmond, 2003).Since algae can utilize $CO_2$for photosynthesis, rise in pH of water during intense photosynthesis is expected (Larsdotter et al., 2007). Besides, the precipitation reactions of cations with P at high pH, algal growth rate and species composition may also be affected by pH, especially in mixed populations in wastewater treatment systems. Though the growth of several algal species have been reported in the range of 7.5 - 8.5 (Marcel et al., 2003; Molina et al., 2001), *Chlorella* sp. in the present study and some other algae (*Rhodobacter sphaeroides*, *Chlorella sorokiniana* and *Spirulina platensis*) (Ogbonna et al. (2000) were able to grow well at higher pH. This shows that *Chlorella* utilized bicarbonate or carbonate as a source of carbon for their growth because of the fact that aquatic cyanobacteria and eukaryotic algae are able to use all forms of dissolved inorganic carbon (free CO2-CO_3-HCO_3) the dominance of which varied depending upon the pH of water. For example, bicarbonate (HCO_3) is most dominant (> 50%) at pH between 6.4 and 10.3, whereas carbonic acid (H_2CO_3) and carbonate (CO_3) are dominant at pH < 6.4 and > 8.3, respectively (Jana 1998; Wetzel, 2006).

It is likely that in *Chlorella* captured carbon is pumped into the cell by bicarbonate transporters present in both the plasma membrane and in the chloroplast envelope as reported for eukaryotic algae (Spalding et al., 2008). Inside the chloroplast, bicarbonate is concentrated, dehydrated spontaneously or by carbonic an hydrase through Calvin-cycle activity, finally yielding algal biomass.

3.3. Growth of *Chlorella* sp. in non autoclaved and autoclaved waste water

There were differences in growth of *Chlorella* between autoclaved and non autoclaved water of a brewery factory. Whereas 50% dilution showed higher growth of *Chlorella* than raw or 25% dilution in non-autoclaved effluent (Fig 6), it was 25% dilution of autoclaved effluent (Fig.7) had the same growth response of Chlorella. This suggests that microbial driven nutrient demandwas reduced by 25% in the autoclaved ones due to death of bacteria in the brewery effluent.

The algal biomass recovered from the wastewater can be used as feedstock for several products including biodiesel, biomethane, fertilizer, and nutraceuticals. Several algal strains have been reported produce neutral lipids (triacylglycerides) that can be converted into biodiesel (Hu et al., 2008; Olaizola, 2003; Schenk et al., 2008; Sheehan, 1998).

4. Conclusion

The conditions prevailed in the standard Bold algal culture medium was adequate for growth of *Chlorella* as no positive impacts of exogenously introduced CO_2 is or aeration were registered. Effect of pH was clearly visible as the growth of *Chlorella* was distinctly higher at pH 8 to 11 compared to pH 4.0- 6.0. Autoclaving of Brewery effluent saved around 25%of the nutrients for chlorella growth.

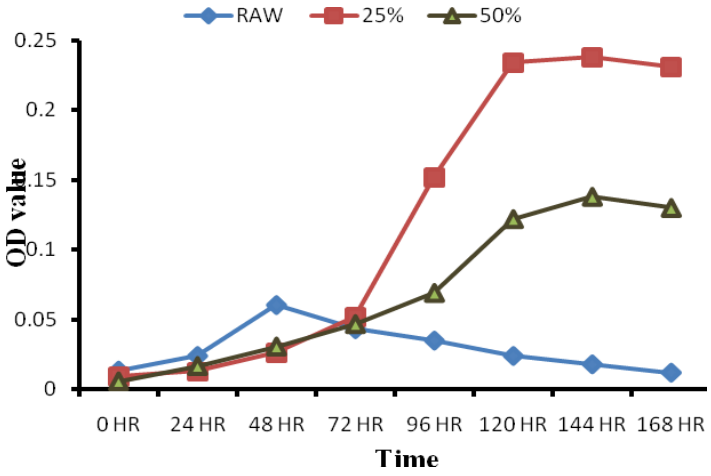


Fig. 7 Growth of *Chlorella* in autoclaved effluent of Beverage factory

References

Acién Fernández FG, Fernández Sevilla JM, Sánchez Pérez JA, Molina Grima E, Chisti Y (2001) Airlift-driven external-loop tubular photobioreactors for outdoor production of microalgae: assessment of design and performance. Chem Eng Sci 56(8):2721-2732.

Cheng L, Zhang L, Chen H, Gao C (2006) Carbon dioxide removal from air by microalgae cultured in a membrane-photobioreactor. Sep Purif Technol 50(3):324-329.

Chisti Y (2008) Biodiesel from microalgae beats bioethanol. Trends Biotechnol 26(3):126-131.

Chiu SY, Kao CY, Chen CH, Kuan TC, Ong SC, Lin CS (2008) Reduction of CO_2 by a high-density culture of *Chlorella* sp. in a semicontinuous photobioreactor. Bioresour Technol. 99(9):3389-3396.

de Morais MG, Costa JAV (2007) Carbon dioxide fixation by *Chlorella kessleri*, *C. vulgaris*, *Scenedesmus obliquus* and *Spirulina* sp. cultivated in flasks and vertical tubular photobioreactors. Biotechnol Lett 29(9):1349-1352.

Eonseon J, Polle JEW, Lee HK, Hyun SM, Chang M (2003) Xanthophylls in microalgae: from biosynthesis to biotechnological mass production and application. J Microbiol Biotechnol 13(2), 165–174

Hosikian A, Lim S, Halim R, Michael KD (2010) Chlorophyll extraction from microalgae: a review on the process engineering aspects. International Journal of Chemical Engineering 2010:1-11

Hu Q, Sommerfeld M, Jarvis E, Ghirardi M, Posewitz M, Seibert M, Darzins A (2008) Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances. Plant J 54(4):621-639.

Jana BB 1998.The State-of-the-art of lakes in India: An overview. Archives fur Hydrobiologie (Suppl) (Monograph Studies) 121: 1 – 89.

Jana BB, Kundu D, Dutta D, Lahiri S, Bag S, Sarkar D, Bhakta JN (2016) Evidences of manure driven and C:N regulated enhanced carbon status and microalgal productivity in managed aquatic system under simulated green house conditions. Journal of Earth Science and Climate Change 7: 336.

Johnson MP (2016) Photosynthesis. Essays Biochem 60: 255–273.

Larsdotter K, Jansen JC, Dalhammar G (2007) Biologically mediated phosphorus precipitation in wastewater treatment with microalgae. Environ Technol 28(9):953-960.

Marcel J, Johannes T, Luuc RM, René HW (2003) Enclosed outdoor photobioreactors: light regime, photosynthetic efficiency, scale-up, and future prospects. Biotechnol Bioeng 81(2):193-210.

Marcel J, Johannes T, Luuc RM, René HW (2003) Enclosed outdoor photobioreactors: light regime, photosynthetic efficiency, scale-up, and future prospects. Biotechnol Bioeng 81(2):193-210.

Molina E, Fernández J, Acién FG, Chisti Y (2001) Tubular photobioreactor design for algal cultures. J Biotechnol 92(2):113-131.

Molina E, Fernández J, Acién FG, Chisti Y (2001) Tubular photobioreactor design for algal cultures. J Biotechnol 92(2):113-131.

Moroney J. V., Ynalvez, R. A., 2010. Proposed Carbon Dioxide Concentrating Mechanism in *Chlamydomonas reinhardtii*. Eukaryotic cell 6 (8), 1251-1259

Nogia P, Kaur S, Mehrotra R, Mehrotra S (2016)Capturing atmospheric carbon: biological and nonbiological methods. International Journal of Low-Carbon Technologies 11(2, 1):266–274.

Ogbonna JC, Yoshizawa H, Tanaka H (2000) Treatment of high strength organic wastewater by a mixed culture of photosynthetic microorganisms. J Appl Phycol 12(3-5):277-284.

Olaizola M (2003) Commercial development of microalgal biotechnology: from the test tube to the marketplace. Biomol Eng 20(4-6):459-466.

Pareek S, Sagar NA, Sharma S, Kumar V, Agarwal T, Gustavo A, Gonzalez-Aguilar, Yahia EM (2017) Chlorophylls: chemistry and biological functions. In: Yahia EM (ed) Fruit and vegetable phytochemicals: chemistry and human health, 2nd edn, Wiley-Blackwell, UK, pp 269-284.

Peterhansel C, Horst I, Niessen MBC, Kebeish R,Kürkcüoglu S, Kreuzaler F (2010) Photorespiration. The American Society of Plant Biologists 8:2-24.

Qiang H, Hugo G, Amos R (1996) A flat inclined modular photobioreactor for outdoor mass cultivation of photoautotrophs. Biotechnol Bioeng 51(1):51-60

Raines CA (2003) The Calvin cycle revisited. Photosynth Res 75: 1-10.

Richmond A, Cheng-Wu Z (2001) Optimization of a flat plate glass reactor for mass production of *Nannochloropsis* sp. outdoors. J Biotechnol 85(3):259-269.

Schenk P, Thomas-Hall S, Stephens E, Marx U, Mussgnug J, Posten C, Kruse O, Hankamer B (2008) Second Generation Biofuels: High-Efficiency Microalgae for Biodiesel Production. Bioenergy Res 1(1):20-43.

Schliep M, Cavigliasso G, Quinnell RG, Stranger R, Larkum AWD (2013) Formyl group modification of chlorophyll a: a major evolutionary mechanism in oxygenic photosynthesis. Plant Cell Environ 36:521–527

Sheehan J, Dunahay T, Benemann J, Roessler P (1998) A Look Back at the U.S. Department of Energy's Aquatic Species Program- Biodiesel from Algae. National Renewable Energy Laboratory (NREL), Golden CO. NREL/TP580-24190. Web. https://www.nrel.gov/docs/legosti/fy98/24190.pdf. Accessed January 17 2016.

Solovchenko A, Khozin-Goldberg I (2013) High-CO_2 tolerance in microalgae: possible mechanisms and implications for biotechnology and bioremediation. Biotechnol Lett 35(11):1745-52.

Solymosi K, Mysliwa-Kurdziel B (2017) Chlorophylls and their derivatives used in food industry and medicine. Mini-Rev Med Chem 17(13):1194-1222.

Ugwu CU, Aoyagi H, Uchiyama H (2007) Influence of irradiance, dissolved oxygen concentration, and temperature on the growth of *Chlorella sorokiniana*. Photosynthetica 45(2):309–311.

Ugwu CU, Aoyagi H, Uchiyama H (2007) Influence of irradiance, dissolved oxygen concentration, and temperature on the growth of *Chlorella sorokiniana*. Photosynthetica 45(2):309–311.

Wetzel R (2006) Transudation of elevated atmospheric CO_2 and natural light trough altered carbon fluxes and higher trophic level production in aquatic ecosystems.Verh. Internat.Verein. Limnol. 29:1112-1128.

Winston MM, Harold HS (1943) Chlorophyll d, a green pigment of red algae J Biol Chem 151:1-19.

Zhang CW, Richmond A (2003) Sustainable, high-yielding outdoor mass cultures of *Chaetoceros muelleri* var. *subsalsum* and *Isochrysis galbana* in vertical plate reactors. Mar Biotechnol 5(3):302-310.