Mapana Journal of Sciences
2019, Vol. 18, No. 2, 1-12
ISSN 0975-3303|https://doi.org/10.12723/mjs.49.1

Mixotrophic Growth of *Chlorella Sp.* Using Glycerol for the Production of Biodiesel: A Review

Sakshi Bansal*

Abstract

Microalgae have great potential for the production of lipids that can be converted into biodiesel. Glycerol is generated as a by-product of the transesterification reaction of the lipid produced by the algae into biodiesel. The process of converting this crude glycerol into the pharmaceutical grade is expensive. Also, glycerol formation from biodiesel production creates surplus glycerol reserves. In this review, the use of crude glycerol as a carbon source for the mixotrophic growth of *Chlorella Sp*. is discussed. Addition of other nutritional sources like nitrogen and phosphorus was also systematically studied and the relationship between the concentration of these nutrients and the growth pattern of the algae was analyzed which is presented in the article as well.

Keywords: Algal Fuel, Mixotrophic Cultivation, Crude Glycerol, Nitrogen Sources, Phosphorus Addition

1. Introduction

As the energy demand around the globe is increasing day by day, thus the need for alternative fuel for energy generation has also increased. Biofuels are an attractive source of energy as they can

* Amity School of Applied Sciences, Amity University, Mumbai, India; sakshi_bansal89@hotmail.com

easily be integrated into our existing systems. Biofuels can be derived from any biomass like plants and algae.

Cultivating crops for extracting biodiesel requires a huge amount of land which creates competition for land with food crops. In 2008, the UK used an estimated 47 billion litres of transport fuel, 53% of which was diesel. If this were met using biodiesel from oilseed rape, it would require 17.5 Mha, more than half the land area of the UK [1]. However, microalgae do not require land and can be grown in brackish or wastewater on wastelands, which relieves the stress on arable land, freshwater, and food production [2]. Thus, extracting biodiesel from microalgae is more advantageous.

Algae strains that are highly productive are usually selected for the conversion of biomass into energy, and strains with relatively high lipid content are highly attractive for biodiesel fuel production.

Microalgae have high growth rates and produce lipids for biofuel production, which in turn decreases the cost of biodiesel production [3]. Microalgae cells can be cultured under autotrophic, heterotrophic and mixotrophic conditions.

The price of the algal biofuel depends upon the cost of the medium used, the yield of lipid, and the quality of the products formed. For mixotrophic and heterotrophic growth, a carbon source is required and the cost of the carbon source represents 50% of the cost of the media used in algal cultivation [4].

Algae can be grown on various carbon sources like glucose, acetate, glycerol or any other organic sources. Glycerol is produced in large quantities as a by-product of biodiesel transesterification and, in 2010; the worldwide production was of about 1.8 billion litres with a commercial demand of only 0.8 million per year. Thus, glycerol is cheaply available and with very poor commercial perspectives. [5] The life cycle analysis of algal biodiesel production is shown in Fig. 1.

2. *Chlorella* Species for Biodiesel Production

Chlorella species are robust and highly productive microorganisms that can grow in different conditions. The quantity of lipid accumulation within the algal cells is different for different strains

and growth media conditions. There are many nutritional and environmental factors controlling the cell growth and its lipid content, such as organic and inorganic carbon sources, nitrogen source, and other essential macro and micro-nutrients like magnesium and copper, temperature, pH level, salinity, agitation speed and aeration (dissolved oxygen), etc. [6].

Chlorella (Chlorophyta, Trebouxiophyceae) is one of the most studied microalgae. Chlorella is one of the algae which are of major interest for producing biofuels, since under stress conditions and depending on the strain, it can accumulate large amounts of lipids [7]. *C. protothecoides* and *C. vulgaris* are two widely available microalgae strains in commercial applications for food and nutritional purposes [8].

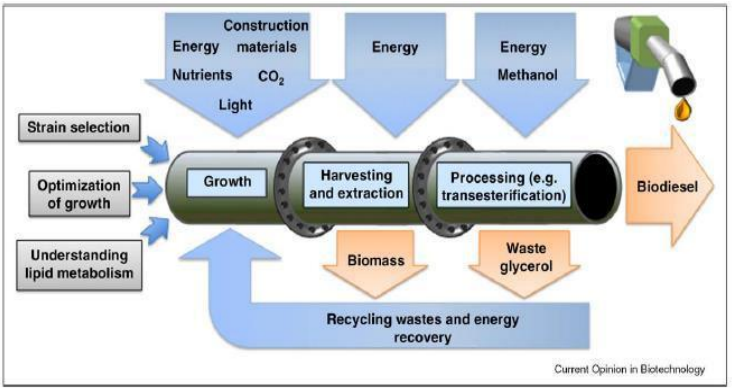

Figure 1: Life cycle analysis of algal biodiesel production [1].

3. Photoautotrophic Cultivation

The photoautotrophic method is the most commonly used and energy saving method of microalgae cultivation. In this process, microalgae convert light and carbon dioxide into chemical energy using photosynthesis. However, autotrophic cultivation has not solved the present need considering volumetric productivities of both biomasses and lipids [9]. As the cells grow, the broth turbidity increases which decreases the light penetration which in turn decrease cell growth and lipid formation. Generally, higher lipid content could be obtained in a nitrogen-limiting or nutrient-

limiting environment; however, the biomass productivity achieved in this stressed condition is usually far lower than that in normal circumstances, which results in unchanged or even lower overall microalgal lipid productivity [2].

4. Heterotrophic Cultivation

In heterotrophic cultivation, the microalgae are grown in the absence of light and an organic compound acts as carbon as well as an energy source. The limitation in autotrophic mode due to light penetration is eliminated here. Heterotrophic algal cultivation has been reported to provide not only high algal biomass productivity but high cellular oil content as well. In the case of *Chlorella protothecoides*, heterotrophic growth on corn powder hydrolysate results in 3.4 times higher biomass yield than that from autotrophic growth while the lipid content increased 4.2 times [10]. Multiple strains of algae have been shown to accumulate lipids to as much as 80% of their dry weight when grown heterotrophically in nitrogen deficient conditions [11]. Heredia-Arroyo et al. reported that the highest lipid concentration in *C. protothecoides* flask cultures was 8.28 g/L using 30 g/L of glucose and 4 g/L of yeast extract as nitrogen supplement. [6] However, heterotrophic cultivation is expensive due to the cost of the substrate and easily contaminated in open systems which cause problems especially in large-scale production [12].

5. Mixotrophic Cultivation

Mixotrophic growth combines photoautotrophic and heterotrophic metabolism which is more useful for overcoming the limitations imposed by photoautotrophic growth. Mixotrophic growth requires relatively low light intensities and, consequently, can reduce energy costs. The high productivity of mixotrophic culture is possibly due to the combined effect of light and organic carbon. An organic nutrient for potential use in commercial scale mixotrophic cultures should be inexpensive, easy to sterilize, promote good growth, and favour the synthesis of the desired by-products [13].

Kong et al. studied *C. vulgaris* on soil extract medium (SEM), and found that the maximal biomass productivity was 654.17 mg/L/day in the culture which had 10 g/L glycerol and 2 g/L glucose, as compared to the control sample (85.42 mg/L/day) and the culture supplied with 5 g/L glycerol and 2 g/L glucose (650.00 mg/L/day) [4].

Heredia-Arroyo et al. investigated *C. vulgaris* grown on the optimized culture medium, cultivated in all the three modes for around 48 h where heterotrophic and mixotrophic modes used 4 g/L of initial glucose concentration. Heterotrophic and mixotrophic microalgal fermentations produced 1.88 and 3.5 times the biomass concentrations of autotrophic cultures, respectively. Autotrophic, heterotrophic and mixotrophic cultures produced similar lipid contents with no significant differences. [8]

Kong et al. compared SEM cultures of *Chlorella vulgaris* in mixotrophic, heterotrophic and photoautotrophic modes. As shown in figure 2, they reported that the biomass contents of mixotrophic and heterotrophic cultures gave a 7.31 and 6.24-fold increase over that in the photoautotrophic, respectively. The maximum specific growth rate (1.08 day-1), biomass productivity (0.35 g/l/day), lipid content (12.64%) and lipid productivity (44.68 mg/l/day) were obtained under the mixotrophic cultivation which was higher than the photoautotrophic and heterotrophic groups [14].

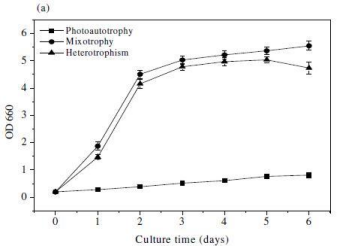

Figure 2: Effect of different nutritional modes on the growth of C. vulgaris in the culture medium [14]

6. Glycerol as a Carbon Source

Glycerol is an inexpensive carbon source that can be used in both heterotrophic and mixotrophic cultivations. Algal growth on pure glycerol and crude glycerol has been examined in numerous studies. Multiple strains of algae, including *Chlorella protothecoides* are able to take and grow rapidly on glycerol.

A comparative study was done by growing *C. protothecoides* cultures on glycerol, glucose and a mixture of glucose and glycerol. There was no significant difference in cell growth and lipid productivities though cultures grown on glycerol exhibited a higher proportion of palmitic and linoleic acids in their lipids while cultures grown on glucose showed a greater proportion of oleic acid in their lipid [11].

Choi et al. investigated the growth of *Chlorella vulgaris* cultivated in Jaworski's medium with glycerol and found the highest biomass concentration in the medium with 5 g/L glycerol was 39.42% higher and the relative Triacylglycerol (TAG) content was 12.20% than that achieved with *C. vulgaris* in the autotrophic medium [3].

Chen et al. compared the growth of *Chlorella protothecoides* in basal culture medium on 30 g glucose/l, pure glycerol and crude glycerol as substrate both in batch as well as fed-batch cultures. In batch cultures, Max biomass concentration with glucose, pure glycerol and crude glycerol as substrates was 15.3 g/l, 19.2 g/l and 23.5 g/l respectively whereas lipid concentration was 7.7 g/l, 9.8 g/l and 14.6 g/l respectively. In Fed-batch cultures, the highest biomass Cell Dry weight (CDW) reached was reported at 46 g/l and lipid content reported was 0.53 g/g CDW, with glucose the biomass CDW reached 43.3 g/l and with lipid content it reached 0.53 g/g CDW in pure glycerol; with biomass CDW it reached 45.2 g/l and with lipid content it reached 0.54 g/g CDW in crude glycerol [15].

7. Nitrogen Supplement

Different nitrogen sources and their concentration have been known to greatly affect the yields of algal lipid. Various nitrogen sources, such as ammonia, nitrate, nitrite, and urea, can be used as

the nitrogen sources for culturing microalgae. Cho et al. reported that when *Chlorella sp*. grown on SEM, was supplemented by 10mM each of ammonia and nitrate showed more lipid production than with urea as a nitrogen source. Also, the maximum biomass obtained from the culture with 0.2 mM ammonia-N was 156.8 mg-DCW/l, while for those with 2 mM nitrogen it was 227.3 mg-DCW/l. However, the maximum lipid production per g DCW with 0.2 mM ammonia-N (143.3 mg) was 2.3 times greater than that with 2 mM ammonia-N (63.0 mg) at day 6 [16].

Shen et al. investigated that for *Chlorella protothecoides* grown on Basal media in the presence of 40g/l glucose, the maximum lipid yield was seen in nitrate media which was at least 103% higher than that in urea media and 38% higher than that in yeast extract media [17] .

Studies also show that nitrogen starvation increases lipid productivity in microalgae. Nigam et al. reported that *Chlorella protothecoides* grown on Fogg's medium with different concentrations of KNO3 in the medium, gave high biomass concentration in high nitrate concentrations but a sharp rise in lipid accumulation of 26% (both in exponential and stationary phase) was recorded when the cultures were grown in initial nitrogen concentration of 0.05g/L KNO3 (1/4th of the original concentration) [18].

Li et al. reported that *C. vulgaris* grown on BG11 medium showed high lipid content (23.9%) in nitrogen-limited (0.1 g L−1 NaNO3) culture with sufficient glucose (10 g L−1 glucose) supply. The highest lipid content of 23.9% (obtained after 240 h culture) was obtained at an initial C/N ratio of 92.7 in culture medium; this compared with 11.3% at C/N ratio 14.0 [19].

8. Phosphorus Supplement

Phosphorus is also essential for the growth of microalgae. Chu et al. studied that when *Chlorella vulgaris* was grown on BG11 media, under nitrogen starvation conditions, the biomass production was enhanced with the increase of phosphorus amount in the medium. This also led to the sharp increase of lipid productivity. The maximum lipid productivity reached 58.4 mg/L/day after 14 days

of cultivation under the highest initial phosphorus content. During the cultivation stage, phosphorus in the liquid medium is transferred and accumulated as Poly-P in cells. Poly-P can serve as an energy source [20].

The same was reported for *Chlorella protothecoides* which was grown on Basal medium by Li et al. Also, the biomass yield with co-deficiency of N and P showed a 7.78-fold decrease than that of control. P-deficiency medium presented higher decrease in biomass yield than N-deficiency medium, which demonstrated that P was more crucial nutritional factor for the growth of *C. protothecoides* [21].

Liang et al. reported that the algal biomass increased when the phosphorus (K2HPO4 or NaNO3) concentration increased in the range from 16 to 80 μM. However, algal biomass was negatively impacted at phosphorus concentrations higher than 80 μM. The lipid content of Chlorella increased with increasing phosphorus concentrations from 16 to 32 μM. However, it was negatively impacted when phosphorus concentration was higher than 32 μM. The maximum lipid content and lipid productivity at the phosphorus concentration of 32 μM were achieved at 23.60 % and 15.67 mg L−1 day−1, respectively [22].

9. Other factors

Blair et al. reported that the growth rate of *Chlorella vulgaris* was higher under blue light as compared to clear, red and green light wavelengths. But blue light required significant time lag to reach exponential growth. Thus, the slow growth rate under blue light is not ideal for practical applications because the faster the exponential phase is reached, the higher will be the biomass production, the shorter will be the pond or photobioreactor volumes for algae production and the shorter will be the harvesting time [23].

Monika Prakash Rai et al. studied the effect of high salinity, light intensity, photoperiod, pH, on growth and lipid production in *Chlorella sp*. on Fogg's medium. The microalga cultures supplied with 0.2 M NaCl showed maximum biomass of 1.021 g L−1 at 360h, whereas concentration above 0.5 M NaCl showed decline in

growth. The highest lipid obtained was 0.0921g L−1 when algae were grown under a red light as compared to yellow, white and green light. *Chlorella sp*. showed maximum lipid production of 0.1995 g L−1 with lipid accumulation of 23% at pH 8 on 24h photoperiod [24].

Yadavalli et al. compared the growth of *Chlorella pyrenoidosa* on different growth mediums (Rudic's Medium, BG11 medium, Modified Basal medium and Chlorella medium) and at different light intensities and found that maximum growth rate was obtained in BG11 medium (6.85 x 106 cells per ml on 14th day) followed by Chlorella medium (5.77 x 106 cells per ml on 14th day) at 55µmol photons m-2 s-1[25].

10. Conclusion

Biodiesel obtained from microalgae has great potential in replacing conventional diesel and reducing the global greenhouse gases emission as well. The big advantage of using microalgae is that they are easily available and can be grown on different substrates and even wastewaters. But we are a long way in reducing the cost of the overall production process and making it available commercially. The focus should be shifted to getting higher lipid yields, reducing the life cycle cost and also bringing it to a commercial scale worldwide.

Mixotrophic growth is a promising method as it requires lesser light intensities and gives high biomass and lipid productivities. Different substrates like glucose, fructose, glycerol, acetate or a combination of them have shown higher biomass and lipid productivities than in photoautotrophic and heterotrophic cultural methods. Crude glycerol is an inexpensive substrate that can be used to grow *Chlorella sp.* Cultures of *C. vulgaris* and *C. protothecoides* showed an increase in growth with the addition of glycerol but up to a limit. A higher level of glycerol had an inhibitory effect on the biomass productivities.

While nitrogen deprivation in *Chlorella sp*. showed an increase in lipid production but the biomass productivities decreased as well, making overall lipid production equal or less than in nitrogen-rich

mediums. Phosphorus-rich medium with nitrogen limitation showed an increase in biomass as well as lipid productivities.

Other factors also affected the growth of the *Chlorella sp*. An optimum pH of about 8 showed an increase in lipid productivity. Adding NaCl supplements also increased the growth of algae but higher salinity caused an inhibitory effect. Light intensities of about 35-55µmol photons m-2 s-1 showed a drastic increase in biomass. Red and blue light gave the highest biomass productivity, yellow and white light also showed an increase whereas green light had an inhibitory effect on the growth.

References

[1] S. A. Scott, M.P Davey, J. S. Dennis, I. Horst, C. J. Howe, D. J. Lea-Smith and A. G. Smith, Biodiesel from algae: challenges and prospects", Current Opinion in Biotechnology, 2010, 277–286.

[2] J. Wang, H. Yang and F. Wang, Mixotrophic cultivation of microalgae for biodiesel production: status and prospects, Appl. Biochem. Biotechnol, 2014, 172: 3307.

[3] H. Choi and S. Yu, Influence of crude glycerol on the biomass and lipid content of microalgae, Biotechnology & Biotechnological Equipment, 2015, 29(3), 506-513.

[4] W. Kong, H. Yang, T. Yun, H. Song, S. Hua and X. Chun-Gu, Effect of glycerol and glucose on the enhancement of bio-mass, lipid and soluble carbohydrate production by *Chlorella vulgaris* in mixotrophic culture", Food Technology and Biotechnology, 2012, 51(1): 62-69.

[5] T. Iago, A. Zouhayr, C. Fábio, O. Carolina, P. Vargas-Machuca, José Antonio, A. P. Fernando, D. Janice Izabel, N. I. Andrade, From waste to energy: microalgae production in wastewater and glycerol, Applied Energy, 2013, 109, 283 – 290.

[6] T. Heredia-Arroyo, W. Wei and B. Hu, Oil accumulation via heterotrophic/mixotrophic *Chlorella* protothecoides, Appl Biochem Biotechnol, 2010, 162, 1978–1995.

[7] A. Guccione, N. Biondi, G. Sampietro, L. Rodolfi, N. Bassi and M. R Tredici, *Chlorella* for protein and biofuels: from strain selection to outdoor cultivation in a Green Wall Panel photobioreactor, Biotechnology for Biofuels, 2014, 7, 84.

[8] T. Heredia-Arroya, W. Wei, R. Ruan, B. Huc, Mixotrophic cultivation of *Chlorella vulgaris* and its potential application for the oil accumulation from non-sugar materials", Biomass and Bioenergy, 2011, 35, 2245-223.

[9] I. O. Ogbonna and J. C. Ogbonna, Effects of carbon source on growth characteristics and lipid accumulation by Microalga Dictyosphaerium sp. with potential for biodiesel production, Energy and Power Engineering, 2018, 10, 29-42.
[10] Y. Liang, N. Sarkany and Yi Cui, Biomass and lipid productivities of *Chlorella vulgaris* under autotrophic, heterotrophic and mixotrophic growth conditions", Biotechnol Lett, 2009, 31, 1043– 1049.
[11] J. O'Grady and J. A. Morgan, Heterotrophic growth and lipid production of *Chlorella* protothecoides on glycerol, Bioprocess Biosyst Eng, 2011, 34, 121–125.
[12] H. M. Amaro, A. C. Guedes and F. Xavier Malcata, Advances and perspectives in using microalgae to produce biodiesel", Applied Energy, 2011, 88, 3402–3410.
[13] C. García, F. G. Camacho, A. S. Mirón, J. M. F. Sevilla, Y. Chisti and E. M. Grima, Mixotrophic Production of Marine Microalga Phaeodactylum tricornutum on Various Carbon Sources", J. Microbiol. Biotechnol., 2006, 16(5), 689–694.
[14] W. Kong, H. Song, Y. Cao, H. Yang, S. Hua and C. Xia, The characteristics of biomass production, lipid accumulation and chlorophyll biosynthesis of *Chlorella vulgaris* under mixotrophic cultivation", African Journal of Biotechnology, 2011, 10(55), pp. 11620-11630.
[15] Y.Chen and T. H. Walker, Biomass and lipid production of heterotrophic microalgae *Chlorella* protothecoides by using biodiesel-derived crude glycerol", Biotechnol Lett., 2011, 33(10), 1973-83.
[16] S. Cho, D. Lee, T. T. Luong, S. Park, Y. Oh and T. Lee, Effects of carbon and nitrogen sources on fatty acid contents and composition in the green Microalga, *Chlorella* sp. 227, J. Microbiol. Biotechnol., 2011, 21(10), 1073–1080.
[17] Y. Shen, Z. Pei, W. Yuan, E. Mao, Effect of nitrogen and extraction method on algae lipid yield, Int J Agric & Biol Eng., 2009, 2(1), 51-56.
[18] S. Nigam, M. P. Rai and R. Sharma, Effect of nitrogen on growth and lipid content of *Chlorella* pyrenoidosa, American Journal of Biochemistry and Biotechnology, 2011, 7(3), 124-129.
[19] C. Li, Y. Yu, D. Zhang, J. Liu, N. Rena and Y. Fenga, Combined effects of carbon, phosphorus and nitrogen on lipid accumulation of *Chlorella vulgaris* in mixotrophic culture", Journal of Chemical Technology and Biotechnology, 2014, 9(3).
[20] F. Chu, P. Chu, P. Cai, W. Li, P. K.S. Lam and R. J. Zeng, Phosphorus plays an important role in enhancing biodiesel productivity of *Chlorella vulgaris* under nitrogen deficiency", Bioresource Technology, 2011, 134, 341–346.

[21] Y. Li, F. Han, H. Xu, J. Mu, D. Chen, B. Feng and H. Zeng, Potential lipid accumulation and growth characteristic of the green alga *Chlorella* with combination cultivation mode of nitrogen (N) and phosphorus (P)", Bioresource Technology, 2014, 174, 24-32.

[22] K. Liang, Q. Zhang, M. Gu and W. Cong, Effect of phosphorus on lipid accumulation in freshwater microalga *Chlorella* sp., J Appl Phycol., 2013, 25, 311–318.

[23] M. F. Blair, B. Kokabian, V. G. Gude, Light and growth medium effect on *Chlorella vulgaris* biomass production, Journal of Environmental Chemical Engineering, 2014, 2, 665–674.

[24] M. P. Rai, T. Gautom and N. Sharma, Effect of Salinity, pH, light intensity on growth and lipid production of microalgae for bioenergy application, OnLine Journal of Biological Sciences, 2015, 15(4), 260-267.

[25] R. Yadavalli, C. S. Rao, D. C. Reddy, K.S.R. Sivasai, S. R. Rao, Effect of different culture media on cell concentrations of *Chlorella pyrenoidosa* under photoautotrophic conditions", International Journal of Natural and Engineering Sciences, 2010, 4(3), 53-57.