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REVIEW PAPER

Investigation of optimal condition for *Chlorella vulgaris* microalgae growth

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ABSTRACT: Due to its abundance and also flexibility of cultivation conditions, *Chlorella vulgaris* microalgae is one of the most ideal options available for microalgae based biodiesel production. Since vulgaris cultivation for fuel production needs economic considerations to be taken, and in first place, the importance of providing biomass and lipid production costs, wide researches have been conducted in this field and this review study aims to spot the best condition for cultivation of this valuable specie by reviewing the whole literature. So far, researchers' efforts show that the best condition for vulgaris cultivation is mixotrophic regime which is done in a bubble column photobioreactor. Glucose as carbonic source and nitrate as nitrogen source have the most efficacy among nutrition conditions. The best results are obtained when glucose and nitrate content are 20 and o.5 g/L respectively. Alkaline medium (pH 9 to 10), non-continuous illumination, 5 to 7 Klux and a 200 mL/min aeration flow rate, were known to be the best physical conditions. The most vulgaris biomass amount produced was 3.43 g/L, and the best lipid productivity was measured 66.25 mg/L/day.

KEYWORDS: *Biodiesel; Chlorella vulgaris; Lipid content; Microalgae growth; Optimum condition*

INTRODUCTION

Clean energy supply has always been a challenge confronting humanity. Long before, fossil fuels were considered as major source of energy, but some reasons such as non-renewability, imminent finishing, pollution, several environmental issues and other harmful effects made man to look for other sources of energy (Gouveia and Oliveira, 2009; Ryan *et al.*, 2006). Wind energy, geothermal energy, solar energy and biofuel are among the most important options proposed during the last century (Khan *et al.*, 2009; Fischer *et al.*, 2001). The biofuel has many unique capabilities that drew attention more than other sources (Erazo*et et al.*, 2007; Golzary *et al.*, 2015). Production sources of biofuel consist of three categories; 1) first- generation fuel sources; food products such as palm oil, sunflower oil, oil seeds such as barley, bran, beets, beans, soybeans and so on, 2) the second-generation fuel sources; fuel-containing cellulose, lignin, or pectin, for example, agricultural residues, 3) the third-generation is the fuel derived from microorganisms such as microalgae and protists (Campbell *et al.*, 2011; Schenk *et al.*, 2008; Fulton *et al.*, 2004; Antolin *et al.*, 2002). Microalgae due to fast growth and ability to cultivate on non-arable land, the use of non-potable water for growing them, independence from seasons, improved efficiency

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of photosynthesis and high lipid content production, flexibility and the ability to modify the conditions of cultivation with the biotechnology techniques, seem to be very desirable (Chisti, 2007; Campbell, 1997; Chisti, 2008; Dalir *et al.,* 2007). However, their application for fuel production on an industrial scale due to the low efficiency of commercially viable oil production is now offline (Benemann, 1997; Sheehan *et al.,* 1998). All the efforts of researchers in recent years are for economizing on fuel production from microalgae. In the first place two parameters including microalgae growth and the produced lipid are taken into account (Cheng *et al.*, 2010; Vicente *et al*., 2004). In this respect microalgae is divided into two categories: The first category has high lipid content but low cell growth, such as botryococcus braunii (Cheng *et al.*, 2007; Cadenas *et al*., 1998) and the second one has a high growth rate but low lipid content, such as *Chlorella vulgaris* (Griffiths and Harrison, 2009). Raised *Chlorella vulgaris* species have attracted special attention. *Chlorella vulgaris* is a photosynthetic microorganisms and eukaryotic from family of chlorellaceae (Ortiz Montoya *et al.*, 2014). This organism is a unicellular green microalgae and has spherical cells with diameter of 2 to 10 micrometers, which has asexual reproduction in which, a mother cell reproduces 4 daughter cells, so that its growth rate is higher (doubling mass cell time is about 19 hours) (Yamamoto *et al.*, 2004; Illman *et al*., 2000; Yamamoto *et al.,* 2005). Rapid growth, easy and flexible terms of culture and resistance against interfering factors, are advantages that makes this microalga appropriate for use in the food industry, aquaculture, cosmetics, pharmaceutical, waste water treatment and the production of biofuel (Hultberg *et al.*, 2014; Bunyakiat *et al.*, 2006). *Chlorella vulgaris* lipid content in typical cultivation condition is about 20% (on dry basis). This amount cannot supply requirements of industrial fuel production but by adjusting the culture conditions which it can be raised to about 40-50% that can be promising for fuel industrial production (Al-lwayzy *et al.*, 2014; Scarsella *et al.*, 2010; Al-Widyan *et al*., 2002). In this study, the major effort was to review and propone the optimum condition for *Chlorella vulgaris* growth with the highest lipid content and also to find the effect of main factors on these parameters. This study has been carried out in the Caspian Faculty of Engineering, College of Engineering, University of Tehran, Rezvanshar, Guilan, Iran in 2016.

Metabolisms of Chlorella vulgaris growth

Microalgae *Chlorella vulgaris* metabolisms are categorized in 4 types, including autotrophic, heterotrophic, mixotrophic and photoheterotrophic. Characteristics of autotrophic microalgae metabolism include using inorganic carbon sources as CO_2 and bicarbonates and light as energy source for photosynthesis (Chenl and Celia, 1994; Demirbas, 2009). This metabolism divided of two categories: open system and close systems. Autotrophic growth with open systems is the most common and cheapest way to produce biomass on a large scale which includes natural pools (such as lakes) and artificial pools (for example containers). The optimum depth of the pond should be between 15-50 cm so that light can reach the whole cultivation environment. In close system for the cultivation of microalgae, several types of photobioreactor, such as tubular, air lift, bubble column and photobioreactors are used (Safi *et al.*, 2014; Pienkos and Darzinc, 2009; Posten, 2009; Molina *et al.,* 2001). Heterotrophic metabolism requires organic carbon as carbonic nutrient and energy in light absence, which its products are produced in closed photobioreactors (Veillette *et al.*, 2012; Huppe and Turpin, 1994). Mixotrophic metabolism is done in the presence or absence of light, with organic and inorganic carbon sources (Chenl and Celia, 1994; Perez *et al*., 2011). Mixotrophic culture means that cell growth is not merely dependent on photosynthesis, light energy is no longer a limiting factor, therefore light and organic carbon source have a supporting role for microalgea (Andrews, 1968; Widjaja *et al.,* 2009). Photoheterotrophic cultivation necessarily takes place in the presence of light and organic carbon source (Chenl and Celia, 1994).

Comparing metabolisms

In autotrophic cultures using the open system, adjusting CO_2 concentration and other parameters such as light intensity is difficult. Using close system (photobioreactors) leads to better management of culture conditions such as light intensity, pH, temperature and CO_2 concentration, but the problem is the complexity of designing closed systems, low-level illumination, high cost of sterilizing and not being economic for industrial applications (Gonzalez *et al.*, 2012; Song and Shi, 2008; Floder *et al*., 2006). Although, heterotrophic compared to autotrophic will result in a higher growth rate but the main problem

is the high cost of organic carbon source and lack of all-time access to it, that limits its applicability in industrial scale (Martinez *et al*., 1991; Liang *et al*., 2009; Ogawa and Aiba, 1981). The major benefit of mixotrophic metabolism is removing waste growth of cell mass duo to dark phase and also decreasing the requirement of organic carbon for growth (De-Bashan *et al*., 2005; Gonzalez and Bashan, 2000). A major barrier to the use of microalgae for biofuel production is reducing the light intensity in dense environment of the culture, which can be solved using mixotrophic operations and the ability of microalgae to grow in presence of organic carbon in dark conditions (Pagnanelli *et al*., 2013). Studies also show that the best conditions for *Chlorella vulgaris* cultivation in order to attain the highest biomass and lipid productivity is achieved under mixotrophic regime (Scarsella *et al*., 2010).

Factors affecting the growth of the microalgae Chlorella vulgaris

Factors affecting the growth of microalgae include two categories: nutritional factors (chemical) and environmental factors (physical). Nutritional factors include the composition and amount of the chemical species in culture medium, the most important them being the source of carbon, nitrogen, phosphorus, silicon, metals such as iron, copper, zinc, vitamins, etc. The type and concentration of the carbon source and nitrogen source are very effective, so they are considered more important and many studies have been done in this area. Of course, there is another parameter that previously was not considered, but it is considerably effective, namely being the concentration of carbon and nitrogen source ratio (C/N) which has many effects on microalgae metabolism. The second, are the physical factors including cultivation environment pH, temperature, light intensity and the intensity of aeration to the system. In the following, we discuss the most important parameters (Faramarzi et al., 2010).

Carbon source

Carbon, the most vital nutrient needed by microalgae, being the main structural component of them and also their energy source. If culture system is autotrophic, CO_2 or bicarbonate compounds (such as sodium bicarbonate) are used as the only carbon source, while in heterotrophic culture, organic materials such as glucose, starch, sucrose, acetate, glycerol, etc play the role of carbon source and in the mixotrophic culture a combination of these twois used. For feeding CO_2 to system, usually a CO_2-enriched air flow with certain percentage of CO_2 is used (such as flue gas stream). It is observed that the change of CO_2 concentration from 4% to 8% and 16% has no significant effect on cell concentration (Wen and Chen, 2001; Xu *et al.,* 2001; Ho *et al*., 2011; Spolaore *et al*., 2006; Hsieh and Wu, 2009; Pyle *et al*., 2008). Morais and Costa (2014) found that the concentration of CO_2 in the medium should be between the value which results in the maximum rate of cell growth and the microalgae tolerance threshold. Montoya *et al.* (2014) implemented the *Chlorella vulgaris* cultivation in an autotrophic tubular photobioreactor, obtaining the highest biomass concentration and the maximum efficiency of lipid production, in air containing 8% CO_2, as 6.8 g/L and 29.56 mg/L/day, respectively. They also found that the concentration of CO_2 affected cellular growth to some extent, but it had no effect on the amount of total lipid. Kong *et al.* (2011) investigated the effect of different carbon sources including CO_2, sodium bicarbonate, sodium acetate, glucose, sucrose and glycerin on the growth of vulgaris. Their results are shown in Fig. 1.

In Fig. 1, the control means CO_2 concentration and OD parameter shown on the vertical axis is abbreviate of optical density and is a method to evaluate the growth of microalgae. According to growth curves obtained in mixotrophic conditions, the optimal carbon source for vulgaris is glucose which its OD curve is higher than others. In this experiment, after 6 days in cultivation environment, SEM (soil extract medium) with glucose concentration of 1 g/L, maximum biomass concentration of 1.23 g/L, the maximum specific growth rate 1.22 1/day and biomass productivity 0.2 g/L/day were obtained. In case of lipid productivity, glucose with 17.3 mg/L/day had the highest yield too. In another experiment, Kong *et al*. (2011) changed glucose concentration from 1 to 20 g/L and found that increasing the concentration of glucose (5 g / L <) may slightly increase the lag phase of cell growth, but after a short interruption, it enters logarithmic phase rapidly and in overall, increasing glucose concentration, increases biomass concentration and amount of lipid cells. Thus, in glucose concentration of 20 g/L, the biomass amount 2.24 g/L and lipid productivity 66.25 mg/L/day were obtained. Scarcella

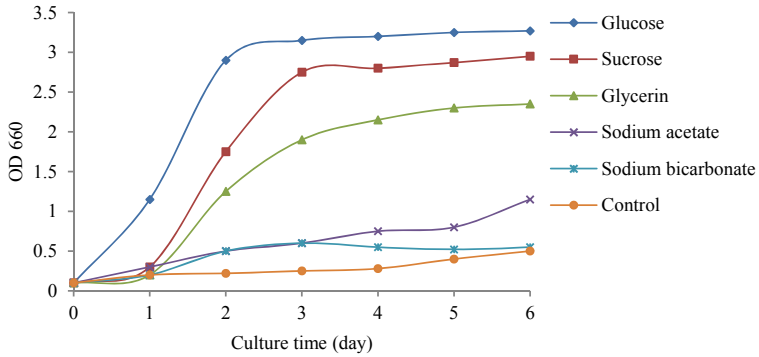

Fig. 1: The effect of different carbon sources on biomass *Chlorella vulgaris* (Kong *et al*., 2011)

et al. (2010) reviewed the optimal growth conditions for vulgaris in bubble column photobioreactor under mixotrophic metabolism and they obtained the optimum concentration of glucose as 6 g/L.

Nitrogen source

In recent years, numerous research works have been performed in increasing the amount of vulgaris lipid cells which most of them has been conducted by focusing on adjustment of nutritional conditions of culture. limitation or lack of nitrogen, phosphate limitation, reducing silicon and the addition of iron were some of them (Rodolfi *et al*., 2009; Liu *et al*., 2008; Griffiths and Harrison, 2009; Yamane *et al*., 2001; Humphrey, 2004). However, the most researches have been performed on nitrogen concentration due to its vital role in regulating cell growth and metabolism of the lipid production. Kong *et al*. (2011) investigated different nitrogen sources for vulgaris including potassium nitrate, urea, ammonium sulfate, ammonium nitrate, peptone, meat extract and simultaneous effect of pH on the growth of the system under mixotrophic conditions that the best results were obtained for potassium nitrate and urea. Among potassium nitrate and urea, potassium nitrate indicated highest specific growth rate (0.87 1/day), biomass production (3.43 g/L), biomass productivity (0.57 g/L/day) and lipid productivity (47.1 g/L/day). But, due to lower price of urea, it was recognized to be the best nitrogen source. In another experiment, different concentrations of urea in range of 0-1 g/L were investigated. Results showed that concentrations greater than 0.5 g/L, although prolong lag phase, but make the logarithmic phase wider, leading to the most growth to be occurred. Totally, it became apparent that limiting the concentration of nitrogen leads to production of more lipids, but on the contrary, it lowers biomass concentration. Li *et al*. (2008) examined the concentration of sodium nitrate in the range 3-20 mM and they achieved highest lipid productivity in 5 mM concentration. Jian-Ming *et al.* (2010) used KNO3 as nitrogen source and found that low amounts of nitrogen (0.2-3 mM) limits the cells growth, and increasing it (to 5 mM) enhances cell growth, as it can be seen in Fig. 2. The highest lipid productivity (40 mg/L/day) was obtained in 1 mM potassium nitrate concentration. Moreover, the amount of lipid productivity in presence of 5 mM KNO3 was obtained 35 mg/L/day. Scarcella *et al.* (2010) investigated the optimal growing conditions for vulgaris in a bubble column photobioreactor under mixotrophic metabolism. They examined the role of nitrogen in two modes: in absence of nitrogen and nitrogen low concentration. In the low nitrogen concentration mode, the highest cell proliferation, biomass production and lipid production was observed.

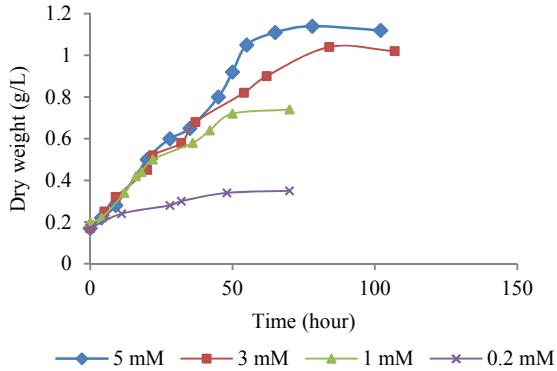

Fig. 2: The effect of different concentrations of potassium nitrate on *Chlorella vulgaris* biomass (Lv *et al*., 2010)

Carbon and nitrogen interaction

A fundamental problem in the optimization of microalgae culture system operating under mixotrophic condition is detailed analysis of organic carbon and nitrogen concentration effect. Several microbiology studies indicated a significant interaction between carbon and nitrogen metabolism in photosynthetic microorganisms (Huppe and Turpin, 1994; Foyer *et al*., 2001; Hilig *et al.*, 2014). Depending on the concentration of nitrogen applied, addition of organic carbon may decelerate or increase the microalgae growth. The amount of organic carbon concentration that will result in least decrease in microalgae growth depends slightly on nitrogen source content and mainly on the concentration of nitrogen. Control of C/N ratio in industrial applications aiming to produce biofuel is essential. At first the high nitrate concentrations are considered in these applications to achieve the desired growth rate and then it is kept at low levels to improve lipid productivity in favorable cellular concentration (Hu and Gao, 2003; Shen *et al.*, 2008; Pilarek *et al.*, 2013; Li *et al.*, 2014; Sayadi *et al.,* 2016). Pagnanelli *et al.* (2013) stated that the mixotrophic growth obeys the interaction between organic carbon and nitrogen. This ratio can have a profound effect on microalgae growth kinetics modeling and culture system operations. Controlling C/N ratio is particularly essential to optimize the performance of the reactor. They presented an exact analysis of interaction effect of organic carbon and nitrogen on specific growth rate. In this study, the average specific growth rate $<\mu_{max}>$ was calculated based on a set of experiments and experimental data. It was observed that increasing concentration of organic carbon, in constant nitrogen concentration, causes a transfer to undesirable growth area (decreasing specific growth rate). It should be noted that in each given nitrogen concentration, there is a maximum concentration of organic carbon, that exceeding it will result in μ_{max} of sample to be lower than $<\mu_{max}>$. This amount also represents entering undesirable growth area and by increasing nitrogen concentration, its amount increases. Thus, it can be concluded that growth regime should be determined using C/N ratio, not carbon or nitrogen concentration alone. The analysis showed evidences that caused avoiding C/N excessive raise. It is known that for C/N above 17, μ_{max} values will be less than $<\mu_{max}>$.

pH

Khalil *et al.* (2010) reported that *Chlorella vulgaris* can grow in a wide range of pH (4-10) and most biomass productivity is achieved in the alkaline environment (pH = 9 and 10). Yu *et al.* (2000) showed that the pH value in the autotrophic growth increased over time as high as 10 but this amount for heterotrophic and mixotrophic growth swayed around 7. Gong *et al.* (2014) investigated the effect of pH using an interesting experiment. In their work, pH was considered in four levels, from neutral to alkaline ranges (7-10). They took two strategies. In the first one, they applied an initial pH, and then applied no control on it to the end of the experiment. In the second strategy, the system started with an initial pH then pH was adjusted to its initial value every day. They found that the most appropriate pH for vulgaris growth is pH range between 10 and 10.5. Also, in pH controlling method, if the pH was in optimal range, the best growth for microalgae will result. Study on the autotrophic cultivation of *Chlorella vulgaris* indicated that there is a complex relationship between CO_2 concentration and pH, which depends on the chemical species equilibrium in the culture system, so as to increase the concentration of CO_2, increases the biomass production. In contrast, decreasing pH may have undesirable effects on cell proliferation (Kumar *et al*., 2010; Yan *et al.*, 2013). Kong *et al.* (2011) research on effect of different sources of nitrogen on changing system pH in mixotrophic *Chlorella vulgaris* culture showed that the use of ammonium, reduced pH to about 3, that was not desired at all, but using potassium nitrate and urea, pH fluctuated about 7.2.

Light intensity

Illumination consists of two subjects: intensity and wavelength of light. Evidence suggests that the light acts as a guide and helping factor to cell proliferation and it helps cellular respiration and photosynthesis. During endothermic reactions for carbon metabolism, energy is needed and this energy is supplied by light. Light is a major factor in the process of photosynthesis to convert carbon dioxide into organic compounds, such as carbohydrates and proteins, in which water and oxygen are released. If the growth of microalgae is done in light limitation, cellular mechanisms progresses to produce carbon into amino acids and other essential compounds for cell, but in the saturated illumination, sugar and starch production

is increased and the maximum growth rate is stabilized. However, some findings suggest a non-continuous illumination strategy, because growth rates remains high and production costs are reduced. This is because cell division for single-celled photosynthetic culture usually occurs under dark conditions. However, for others cases, cell division occurs in both dark and light phases, but for vulgaris microalgae, more cell division occurs after stopping the lighting phase. Moreover, some enzymatic mechanisms may be disabled during illumination (Hultberg *et al*., 2014; Al-Qasmi *et al*., 2012). A research under different light intensities of approximately 3, 5 and 7 kLux and 8:16, 12:12 and 16:8 lighting periods (light:dark) was performed to investigate the matter (Zehnder and Gorham, 1960; Mayo, 1997). Fig. 3 shows such a long time lighting (16:8) which will increase vulgaris biomass, and the maximum biomass (2.025g/L) is obtained in 5 kLux and lighting period of 16:8. In Fig. 3 under high light intensity conditions, 7 kLux, creation of light barrier, reduces the production of biomass, because the extra light can not be absorbed for photosynthesis and it may damage to microalgae and stop its growth (Khoeyi *et al*., 2012; Ryu *et al*., 2009).

Studies showed that lipid and PUFAs (poly unsaturated fatty acids) decrease with increasing light intensity (Orcutt and Patterson, 1984; Corre *et al*., 1996). Light application efficiency may be optimized by prolonging the period of darkness under high light intensity. This allows the photosynthetic activities in cells to use all available photons and convert them into chemical energy, thus it prevents inhibiting effects of light (Long *et al*., 1994). Also in Table 1, the percentage of SFA, MUFA and PUFA respectively, saturated fatty acid which is suitable for biodiesel production, monounsaturated fatty acid and polyunsaturated fatty acid are shown in different light regimes. With increasing light intensity, the amount of SFA increases and the amount of MUFA and PUFA reduce (Richmond *et al*., 2004; Ugwu and Aoyag, 2012). In another experiment, the cultivation illumination was set at 3, 7 and 14 kLux levels. As Fig. 4 indicates for vulgaris in 7 days of culture, light intensity of 14 kLux is optimum, but at more cultivation time like 17 days, 7 kLux was optimal condition. In another study maximum lipid of 20% and the maximum concentration of biomass, 0.75

Table 1: Fatty acid composition of *Chlorella vulgaris* in different light regimes (Richmond *et al*., 2004)

Light Regime	2800(Lux)			4600(Lux)			7400(Lux)		
	8:16h	12:12h	16:8h	8:16h	12:12h	16:8h	8:16h	12:12h	16:8h
Saturated fatty acid (SFA)									
14:0	0/6±0/2	0/7±0/3	0/8±0/2	0/8±0/4	1/22±0/5	1/6±0/3	1/5±0/4	1/2±0/2	1/5±0/4
16:0	20/22±1/2	21±1/5	21/4±0/4	22/1±2/5	22/1±0/3	22±3/5	21/5±0/3	21/5±0/4	21/7±0/7
18:0	3±0/3	3/4±0/4	3±0/1	3/6±0/4	4/1±0/5	4/25±0/6	6/5±0/5	6/5±0/7	6/4±0/6
20:0	0/11±0/1	0/2±0/1	0/22±0/1	0/1±0/1	0/15±0/1	0/25±0/2	0/2±0/1	0/23±0/2	0/19±0/1
24:0	2±0/5	3±0/4	3/25±0/3	3±0/4	3/5±0/2	3/13±0/5	3±0/4	3/95±0/7	4±0/3
Total SFAS	25/9^{C}	28/25^{d}	28/67^{bc}	29/6^{cd}	31/1^{b}	31/23^{cd}	32/7^{ab}	33/38^{a}	33/38^{a}
Momounsaturated fatty acid (MUFA)									
14:ln-5	0/7±0/2	0/1±0/1	0/4±0/2	0/21±0/2	0/38±0/2	0/56±0/3	0/47±0/2	0/49±0/3	0/52±0/4
16:ln-7	0/7±0/2	0/8±0/3	0/6±0/3	0/7±0/4	0/36±0/2	0/4±0/2	0/42±0/3	0/38±0/2	0/35±0/2
18:ln-9	12±3	12±4	12/1±4/5	12/05±5/2	11/56±5	11±6	10/99±5	10/8±4/6	10/7±0/3
19:ln-9	2/03±0/5	0/9±0/3	1/45±0/5	1/16±0/4	0/53±0/2	0/84±0/5	0/69±0/4	0/77±0/5	0/73±0/3
20:ln-9	0/5±0/2	0/6±0/4	0/6±0/3	0/29±0/2	0/32±0/2	0/35±0/3	0/34±0/2	0/34±0/1	0/3±0/2
Total MUFAs	15/93^{a}	14/33^{ab}	15/15^{ab}	14/41^{ab}	13/15^{bc}	13/15^{cd}	12/9^{dc}	12/78^{c}	12/6^{f}
Polyunsaturated fatty acids (PUFA)									
18:3n-3	18/25±6	18±5	18±4/5	19±6	19/4±5/5	19/38±0/5	18/4±4	18/3±5/5	18/1±3
18:3n-6	4/45±1.5	4/1±0/2	4±1/2	3/7±1	3±1/5	2/38±1	1/8±0/5	1/5±1	1/3±0/5
20:2n-6	0/34±0/2	0/23±0/1	0/1±0/1	0/11±0/1	0/12±0/1	0/08±0/05	0/1±0/05	0/09±0/4	0/08±0/02
20:4n-6	0/98±0/2	0/85±0/3	0/9±0/2	0/8±0/3	0/72±0/4	0/7±0/4	0/35±0/2	0/3±0/1	0/25±0/1
20:5n-3	0/88±0/2	0/87±0/3	0/88±0/4	0/87±0/4	0/58±0/2	0/28±0/1	0/2±0/1	0/2±0/1	0/2±0/1
22:5n-6	2±0/5	2±1	1/3±0/3	1/1±0/5	0/8±0/3	0/95±0/4	0/6±0/2	0/45±0/3	0/1±0/1
22:6n-3	0/5±0/1	0/58±0/2	0/4±0/2	0/4±0/3	0/85±0/4	071±0/3	0/25±0/2	0/15±0/1	0/3±0/1
Total PUFAs	27/4^{a}	26/63^{ab}	25/58^{b}	25/98^{ab}	25/47^{b}	24/48^{b}	21/7^{b}	20/99^{b}	20/33^{b}

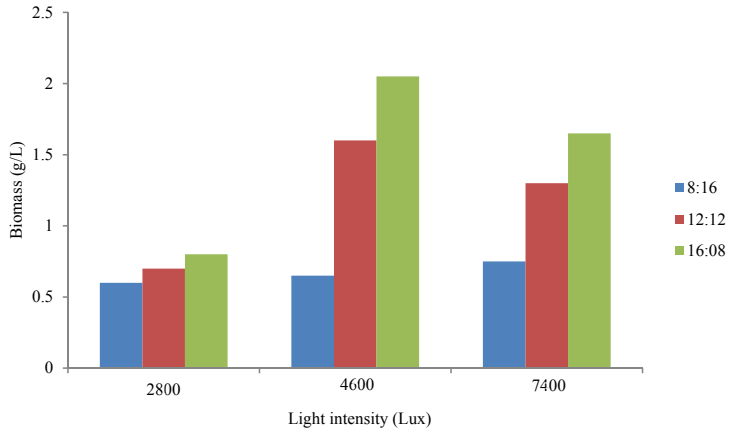

Fig. 3: *Chlorella vulgaris* biomass in the three periods and three different light intensities (Brody and Vatter, 1959)

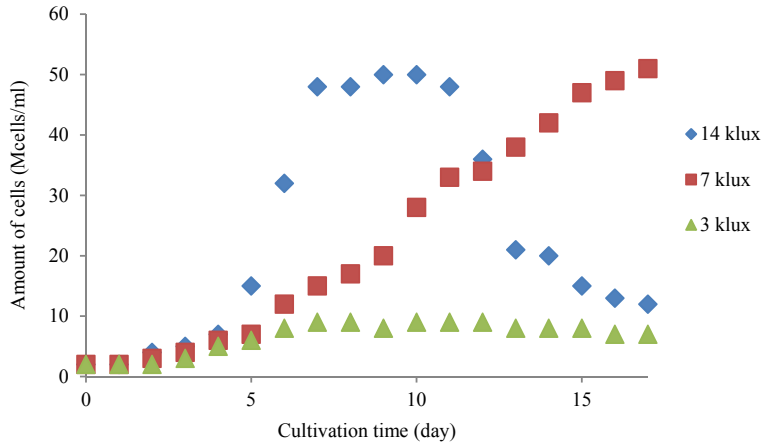

Fig. 4: Growth of biomass at different levels of lighting (Lv *et al*., 2010)

g/L were obtained in 14 kLux, while cells under 2 and 9 kLux of light, achieved the amount of lipid 14.1% and 11%, respectively (Dvoretsky *et al*., 2015).

However, in the case of wavelength, microalgae usually uses wavelengths between 400-700 nm for photosynthesis. The wavelength microalgae absorbs varies depending on the type of microalgae (Blair *et al*., 2014). Maximum and minimum number of *Chlorella vulgaris* cells production is in red light (λ=630-665nm) and blue light (λ=430-465) respectively. Mathy *et al.* (1996) reported that red light leads to increase chlorophyll pigment, which reflected the positive effect of red light (Wang *et al*., 2007; Yek and Chang, 2011). Vulgaris cell size measurement revealed that the growth of microalgae cells under blue light, compared to red light had an approximately increase of 70-60% in diameter (Fig. 5).

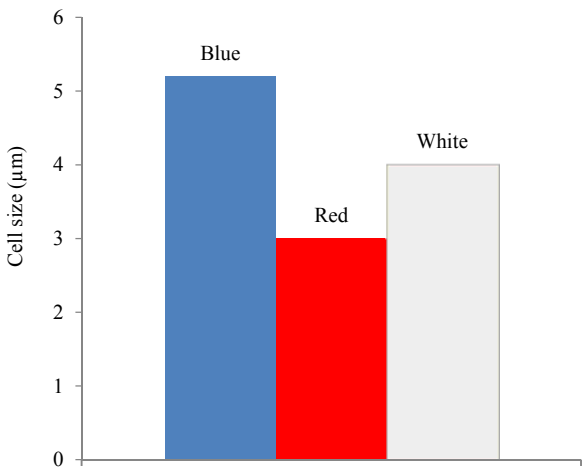

Fig. 5: The effect of different wavelengths on cell size (Wang *et al*., 2007)

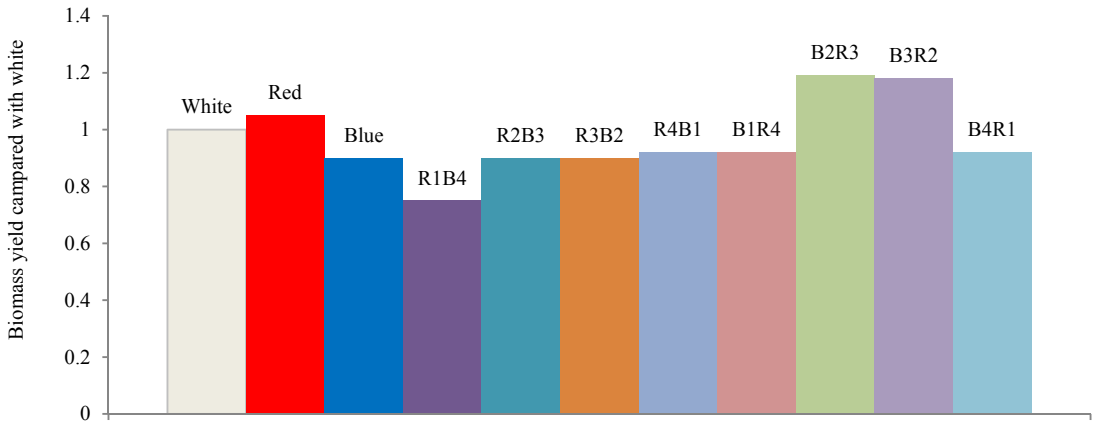

Fig. 6: Biomass production obtained with monochromatic lights and the combine wavelengths (Wang *et al*., 2007)

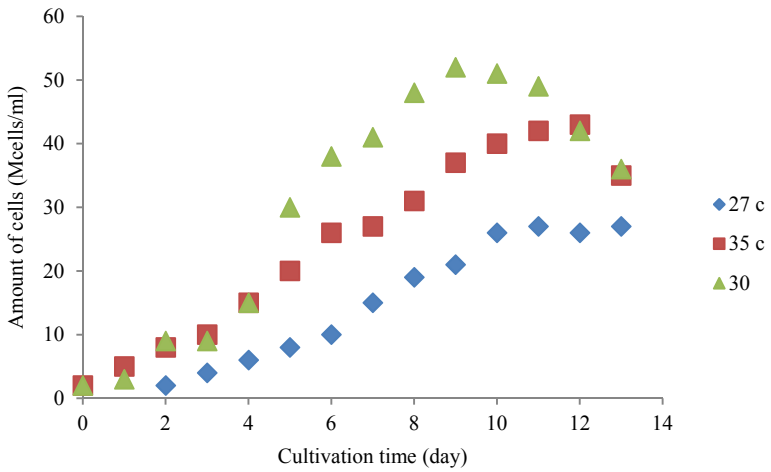

Fig. 7: Growth of biomass at different temperatures (Dvoretsky *et al*., 2015)

Fig. 6 shows that the blue light is not effective for growth alone. The best condition for maximum biomass is B3R2 with 3 days blue and 2 days red and B2R3 with 2 days blue and 3 days red. Because the initial blue light creates a large cell size and high potential for secondary divisions under red light and also increases the final products (Kim *et al*., 2014).

Temperature

One of the most important environmental factors affecting various aspects of growth and fatty acid composition for several microorganisms is temperature. Temperature also can affect enzymatic reactions, cell membrane system and other characteristics (Zeng *et al*., 2011). Growing conditions at low temperature leads to a spontaneous reaction and change the cellular mechanism and thereby reduction in the fluidity of the cell membranes. This will increase the proportion of unsaturated fatty acids to compensate for fluidity reduction. Low temperature limits cell growth speed and therefore reduces the biomass production (Nishida and Murata, 1996). The optimal temperature for *Chlorella vulgaris* is about 30°C, in which the maximum biomass productivity is achieved (Chinnasamy *et al*., 2009; Xu *et al*., 2006). Converti *et al*., (2009) reported that *Chlorella vulgaris* growth rate at 35°C decreases 17% compared to 30°C. An excessive rise in temperature to 38 ° C leads to an abrupt halt in microalgae growth and cells die. With increasing temperature to 30 ° C cell growth

rate increases and then decreases with increasing temperature to 35°C (Cassidy, 2011). Similar results show that the highest biomass efficiency is obtained at 30±2°C and with increasing temperature to 35±2, the efficiency of biomass drops (Barghbani *et al*., 2012). Dvoretsky *et al.* (2015) has reported that the highest biomass production, 51 Mcell/ml was achieved in 9 days of culture at 30 ° C (Fig. 7).

In another study after 7 days of culture, optimum temperature has been reported between 30-35 ° C and the most biomass was obtained 3.6 g/L (Barghbani *et al*., 2012). Temperature increases in *Chlorella vulgaris* from 25-30°C reduces the amount of lipid percent from 14.7% to 9.5% (Converti *et al*., 2009) and also protein synthesis (Konopka and Brock, 1978). An increase in temperature from 20 to 30°C, increases the concentration of intracellular free amino acids from 840 to 1810 mg (per 100 g dry weight), and is followed by reduction in protein and starch amount (Nakamura and Miyachi , 1982; Mitsui *et al*., 1977).

Aeration

Aeration is one of the key parameters for microalgae culture medium and depending on the microalgae species, the type of culture of open system or photobioreactor and culture system scale (big or small), there are several ways to apply it (Ugwu and Aoyagi, 2012). The benefits of aeration or mixing include preventing precipitation of microalgae, homogenization of cultivation environment so that all of the cells can reach light and food, avoiding temperature differences (isothermal condition through the cultivation environment) and facilitating the exchange of gases between the cultivation environment and air. Proportional to the scale of culture, mixing may be done by manual shaking on a daily basis for laboratory scale cultivation in erlenmeyer flasks and test tubes, or aerated peristaltic pump for larger scale or use the circulator arms in large pool (Lavens and Sorgeloos, 1996; Morric *et al*., 2008). Liang *et al*. (2009) stated that the maximum amount of cells lipid with aeration intensity of 200 ml/min was obtained 54 mg/L (Ogawa and Aiba, 1981). Khoeyi *et al*. (2012) obtained the maximum concentration of biomass 2.05 g/L in aeration intensity of 3 cm^3/s. Kim *et al.* (2014) reached cellular fatty acid content of 11.07% (on dry basis) with air flow rate of 100 ml/min. Although aeration is an important factor in biomass growth, especially at the starting time of the formation of the first cellular nucleus and severity of aeration or unbalanced aeration can lead to first cellular nucleus death. But, so far the direct effect of aeration on growth and cellular lipid production rate has been less studied and is often intended as a fixed side factor (Kim *et al*., 2014).

Biodiesel production from Chlorella vulgaris

After microalgae cultivation, there are several downstream processes to biodiesel production including: 1) Harvesting/dewatering: since algal cultures are mainly grown in water it is required to concentrate harvested algal biomass prior to extraction and conversion. 2) Extraction: next step is extraction of lipids including triglycerides and fatty acids from algal biomass. It can be performed by different methods but most common methods are Folch and Bligh-Dyer. 3) Conversion: final process is conversion of lipid to methyl ester fatty acids. This reaction is done between lipid and methanol in presence of alkaline catalyst and products are glycerol and methyl esters that can be used as biodiesel (Blinova *et al*., 2015; Faramarzi *et al*., 2010). Miao and Wu (2006) expressed that produced biodiesel of *Chlorella vulgaris* has 39 MJ/kg heat value equal to its heat of combustion. Cao *et al.* (2013) obtained the biodiesel production efficiency about 92% using vulgaris.

CONCLUSION

Currently biofuel with microalgae origin due to environmental and economic capabilities is one of the most important human options for the supply of clean, inexpensive and reliable energy. For this purpose, different species of microalgae have been studied and evaluated. Among them, *Chlorella vulgaris* due to their favorable biological characteristics are highly regarded. The growth of these microorganisms under different growth conditions and regimes is one of these features, but overall research proved that its culture under the simultaneous presence of light and an organic carbon source (mixotrophic culture), will result in highest biomass productivity and cellular lipid to produce biodiesel. The optimal condition for growth, through nutritional conditions, carbon source and nitrogen source is most effective. Based on empirical studies conducted, best carbon source for vulgaris is glucose and its optimal concentration is obtained 20 g/L with the largest biomass production of 2.24 g/L, and the maximum lipid productivity of

Table 2: Summary of the *Chlorella vulgaris* growth studies

Process parameters	Chlorella vulgaris growth items	References
Growth metabolism	Best metabolism for vulgaris growth is mixotroph	Scarcella *et al.*, 2010 Pagnanelli *et al.*, 2013
Carbon source	Best carbon source indicated glucose with 20 g/L concentration and most Biomass production 2.24 g/L	Kong *et al.*, 2011 Scarcella *et al.*, 2010
Nitrogen source	Best nitrogen source indicated nitrate with 0.5 g/L concentration and most Biomass production 3.43 g/L	Kong *et al.*, 2011 Jian-Ming *et al.*, 2010
C/N ratio	It must not be more than 17	Pagnanelli *et al.*, 2013 Li *et al.*, 2014
pH	Alkaline environment with pH=9-10 obtained best result and most cell density about 16 Mcell/ml	Khalil *et al.*, 2010 Gong *et al.*, 2014
Light intensity	Optimum light intensity reported 5 kLux with most biomass production 2.025 g/L	Khoeyi *et al.*, 2012 Ryu *et al.*, 2009
Temperature	Optimum temperature indicated 30 ^{0}C with most biomass production 3.6 g/L	Barghbani *et al.*, 2012 Conoverti *et al.*, 2009
Aeration	Aeration intensity 100 ml/min obtained best result and most biomass production 2.05 g/L	Khoeyi *et al.*, 2012 Kim *et al.*, 2014

66.25 mg/L/day. Among the sources of nitrogen, nitrate compounds showed the best results. Thus, the maximum biomass production and high lipid productivity was obtained in concentration of 0.5 g/L potassium nitrate, 3.43g/L and 47.1 mg/L/day. The relationship between the concentration of carbon and nitrogen sources, existence of interaction in form of the C/N ratio is clearly proven, but more accurate analysis and explanation requires further researches. The best physical condition in order to achieve maximum growth performance and lipid production was obtained in alkaline environment (pH=9 to10), temperature 30°C, light intensity 5-7 kLux and aeration intensity 100 ml/min respectively. The summary of researchers on *Chlorella vulgaris* growth is shown in Table 2.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this manuscript.

ABBREVIATIONS

CO_2	Dioxide carbon
C/N	Carbon nitrogen ratio
SEM	Soil extract medium
KNO3	Nitrate potassium
μ_{max}	Average specific growth rate
PUFA	Polyunsaturated fatty acid
SFA	Saturated fatty acid
MUFA	Monounsaturated fatty acid
λ	Wavelength

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