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 in order to product of microalgae based biodiesel. Since vulgaris cultivation for fuel production needs economic considerations to be taken, and in first place providing biomass and lipid production costs is important, 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 research conducted. 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. It is known the best results obtain in amounts glucose and nitrate of 20 and 0.5 g/L respectively. Alkaline medium (pH 9 to 10), non-continuous illumination, 5 to 7 Klux and a 200 mL/min aeration flow rate, indicated 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.

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**RUNNING TITLE:** Optimal condition for *Chlorella vulgaris*

**INTRODUCTION**

Clean energy supply has always been a challenge confronting humanity. Long before, fossil fuels were considered major source of energy, but some reasons such as nonrenewability, 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 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 al., 2007; Golzary et al., 2015). Production sources of biofuel consists 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 of the seasons, improved efficiency 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., 2017). 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 has 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, such advantages makes this microalgae 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% (based dry weight). This amount cannot supply requirement to fuel industrial 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-Iwayzy et al., 2014; Scarsella et al., 2010; Al-Widyan et al., 2002). In this study, the maximum effort was to review and propose the optimum condition for Chlorella vulgaris growth with the highest lipid content and also finding 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 is divided into 4 types, including autotrophic, heterotrophic, mixotrophic and photoheterotrophic. Metabolism characteristic of autotrophic microalgae is inorganic source of carbon as CO2 and bicarbonate and light as an energy source that uses light and inorganic carbon to photosynthesis (Chenl and Celia, 1994; Demirbas, 2009). This metabolism divided of two categories; open 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 in order to light influence over the medium well done. In the close system for the cultivation of microalgae types of photobioreactor, such as tublar, air lift, bubble columns photobioreactor are used (Safi et al., 2014; Pienkos and Darzinc, 2009; Posten, 2009; Molina et al., 2001). Heterotrophic metabolism requires organic carbon as carboxic nutrient and energy source with no light that its products are produced in close photobioreactors (Veillette et al., 2012; Huppe and Turpin, 1994). Mixotrophic metabolism in the presence of light or without light made with organic and inorganic carbon source (Chenl and Celia, 1994; Perez et al., 2011). Mixotrophic culture means that cell growth is strongly dependent on photosynthesis, light energy is no longer a limiting factor, therefore light and organic carbon source have a supporting role for microalgae (Andrews, 1968; Widjaja et al., 2009). Photoheterotrophic cultivation necessarily take place in the presence of light and organic carbon source (Chenl and Celia, 1994).

Compare the types of metabolisms

In autotrophic cultures using the open system, adjust CO2 concentration and other parameters such as light intensity is difficult. Using close system (photobioreactors) leads to better management culture conditions such as light intensity, pH, temperature and CO2 concentration, but the problem is the complexity of designing closed systems, low-level illumination, high cost of sterilizing and not economic for industrial applications (Gonzalez et al., 2012; Song and Shi, 2008; Floder et al., 2006). Although, heterotrophic compared with autotrophic will result a higher growth rate but the main problem is the high price of organic carbon source and lack of regular access to it that its use limits on industrial scale (Martinez et al., 1991; Liang et al., 2009; Ogawa and Aiba, 1981). The major benefit of mixotrophic is removing waste growth of cell mass due 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 the production of biofuel is reducing the intensity of light in the high dense of the culture which mixotrophic operations can use the power of microalgae to grow in the presence of organic carbon in dark conditions to solve this problem (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 microalgae Chlorella vulgaris

Factors affecting the growth of microalgae can be divided into two categories of nutritional factors (chemical) and environmental factors (physical). Nutritional factors include the composition and amount of the chemical species present in culture medium which the most important of these are 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 more attention attracted them 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 and that is ratio of the concentration of carbon and nitrogen sources (C/N) which can affect much microalgae metabolism. The physical factors that some of the most important of them are pH culture, temperature, light intensity and the intensity of aeration to the system. In later on, the most important of these parameters will be discussed.

Carbon source

Carbon, the most basic requirements of food microalgae, which is the main ingredient structure of the building and their energy source. If culture system, is autotrophic CO₂ or bicarbonate compounds as example sodium bicarbonate uses as the carbon source while in heterotrophic culture, necessarily organic materials such as glucose, starch, sucrose, acetate, glycerol, etc, play the role of carbon source and in the mixotrophic culture is used combination of these two. For feeding CO₂ to system usually uses a CO₂-enriched air flow with certain percentage of CO₂ such as flue gas stream. It is observed that the change of CO₂ 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 CO₂ in the medium should be not lower than the value will result the maximum rate of cell growth and should not be above the threshold of tolerance microalgae. Montoya et al. (2014) showed the Chlorella vulgaris cultivation in an autotrophic tublar photobioreactor, with the highest concentrations of bio-oil production and maximum efficiency in air containing 8% CO₂ by as much as 6.8 g/L and 29.56 mg/L/day, respectively. They also found that the concentration of CO₂ will have no effect on cell proliferation too which had no significant influence on the amount of total lipid. Kong et al. (2011) investigated the effect of different carbon sources, including CO₂, sodium bicarbonate, sodium acetate, glucose, sucrose and glycerin on the growth of vulgaris. Their results are shown in Fig. 1.

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

In Fig. 1, control means CO₂ concentration and OD parameter shown on the vertical axis is abbreviated 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 it is OD curve is higher than others. In this study, after 6 days in soil extract medium (SEM) culture with glucose concentration 1
g/L, maximum biomass concentration 1.23 g/L, the maximum specific growth rate 1.22 1/day and biomass productivity 0.2 g/L/day obtained. In the case of lipid productivity as well as glucose with 17.3 mg/L/day had the highest yield. In another study, Kong et al. (2011) change glucose concentrations from 1 to 20 g/L and found that increasing the concentration of glucose (5g/L <). However, it may slightly increases the lag phase of cell growth but after a short interruption it entered logarithmic phase rapidly and in overall, increasing glucose concentration, increases biomass concentration and amount of lipid cells. Thus, in glucose concentration 20 g/L, the biomass 2.24 g/L and lipid productivity 66.25 mg/L/day was obtained. Scarcella et al. (2010) reviewed the optimal growth conditions for vulgaris in bubble column photobioreactor under mixotrophic metabolism and he obtained optimum concentration of glucose 6 g/L.

**Nitrogen source**

In recent years, numerous research works have been performed in increasing the amount of vulgaris cells lipid which most of them has conducted by focusing on the nutritional conditions adjustment of culture. For instance, due to the limitation or lack of nitrogen, phosphate limitation, reducing silicon and the addition of iron (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 obtained for potassium nitrate and urea. Between 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, the nitrogen source recognized to be better than any other sources. In other study, different concentrations of urea contain 0-1 g/L was investigated. Results showed that concentrations greater than 0.5 g/L, although yield the longer lag phase but it make the more vast logarithmic phase and more growth will occur. In total, it became apparent that limiting the concentration of nitrogen leads to the production of more lipids, but on the contrary, it makes the lower biomass. 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 (mM 0.2-3) limit the cells growth, and by increasing the amount of 5 mM enhanced cell growth where can be seen in Fig. 2. The highest lipid productivity to (40 mg/L/day) obtained in 1 mM potassium nitrate concentration. Moreover, the amount of lipid productivity for 5 mM KNO3 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 tested the role of nitrogen in two modes without adding nitrogen and nitrogen limit concentration which in the limited 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 systems that are operated 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, organic carbon addition may be decelerate or contributed to the growth of microalgae. The amount of organic carbon concentration that will result least deceleration growth has depended slightly to nitrogen source used and mainly to the concentration of organic carbon is applied. Control of C/N ratio in industrial applications to produce biofuels 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 obtained (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 was obeyed the interaction between organic carbon and nitrogen. It can have a profound effect on microalgae growth kinetics modeling and culture system operations. Control of 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 set of experiments and experimental data. It was observed that increasing of concentration of organic carbon while the nitrogen concentration is kept at constant unchanged, causes the transfer to an undesirable growth area at the lower specific growth rate. It should be noted that in each nitrogen concentration, there is a maximum concentration of organic carbon that for its higher values, $\mu_{max}$ of sample will be less than $<\mu_{max}$. This amount also represents to enter undesirable growth area and by increasing nitrogen concentration, its amount increases. Thus, it can be concluded that growth regime should determine with the C/N ratio support, not carbon concentration or nitrogen concentration alone. The analysis showed evidences that cause avoiding C/N excessive rise. It is known that for C/N more than 17 $\mu_{max}$ 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 (pH = 9 and 10). Yu et al. (2000) showed that the pH value in the autotrophic growth increased over time and it achieved to about 10 but this amount for heterotrophic and mixotrophic growth swayed around 7. Gong et al. (2014) investigated the effect of pH by interesting experiment. In their work, pH at four levels neutral to alkaline ranges (7-10) was considered. They have two strategies to work. In the first, it was applied an initial pH, and then to the end of the experiment, system pH was not controlled. In the second method, the system started with an initial pH then daily pH adjusted to its initial value. They found that the most appropriate pH for vulgaris growth is pH range between 10 and 10.5. Also, second method of controlling the pH, if the pH be 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 present in the culture system, so as to increase the concentration of CO$_2$, increases the biomass production. In contrast, the decrease in pH can have undesirable effects on cell proliferation (Kumar et al., 2010; Yan et al., 2013). Kong et al. (2011) research by mixotrophic Chlorella vulgaris culture on the effect of using different nitrogen sources in changing system pH showed that the use of ammonium, reduced pH to about 3 that was not desired at all, but about 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 guide and helper of cell proliferation and it helps the effect on cellular respiration and photosynthesis. During endothermic reactions for the metabolism of carbon, energy is needed and the 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 to be done in the 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 increased and the maximum growth rate stabilized. Although some findings suggest a non continuous illumination
strategy because growth rates remain high and production costs are reduced. This is because cell division for more single-celled photosynthetic culture occurs under dark conditions, however, for others, cell division occurs in both the dark and light phases, but for microalgae vulgaris, more cell division is after stopping the lighting phase. Moreover, some enzymatic mechanisms may be disabled during illumination (Hultberg et al., 2014; Al-Qasmi et al., 2012). Research under different light intensities of approximately 3, 5 and 7 kLux and photoperiod 8:16, 12:12 and 16:8 (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) achieves in 5 kLux and photoperiod of 16:8. In Fig. 3 under high light intensity conditions, 7 Klux, creation of light barriers, reduces the production of biomass because the extra light cannot be absorbed for photosynthesis and it may damage to microalgae and stop its growth (Khoeyi et al., 2012; Ryu et al., 2009).

Studies show that lipid and PUFAs as poly unsaturated fatty acids reduced 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 allowed the photosynthetic activities in cells for full use of available photons and convert them into chemical energy thus prevents inhibiting effects of light (Long et al., 1994). Also, in Table 1, the percentage of SFA, MUFA and PUFA respectively, saturated fatty acid that are 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 increase 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, 20% and the maximum concentration of biomass 0.75 g/L obtained in 14 kLux, while 2 and 9 kLux, achieved the amount of lipid 14.1% and 11%, respectively (Dvoretsky et al., 2015).

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

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

<table>
<thead>
<tr>
<th>Light Regime</th>
<th>2800(Lux)</th>
<th>4600(Lux)</th>
<th>7400(Lux)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>8:16h</td>
<td>12:12h</td>
<td>16:8h</td>
</tr>
<tr>
<td>Saturated fatty acid (SFA)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14:0</td>
<td>0/6 ± 0/2</td>
<td>0/7 ± 0/3</td>
<td>0/8 ± 0/2</td>
</tr>
<tr>
<td>16:0</td>
<td>20/22 ±</td>
<td>21 ± 1/5</td>
<td>21/4 ± 0/4</td>
</tr>
<tr>
<td></td>
<td>18:0</td>
<td>20:0</td>
<td>24:0</td>
</tr>
<tr>
<td>-------</td>
<td>------</td>
<td>------</td>
<td>------</td>
</tr>
<tr>
<td></td>
<td>3±0.3</td>
<td>0/11±0.1</td>
<td>2±0.5</td>
</tr>
<tr>
<td></td>
<td>3/4±0.4</td>
<td>0±2±0.1</td>
<td>3±0.4</td>
</tr>
<tr>
<td></td>
<td>3±0.1</td>
<td>0/22±0.1</td>
<td>3/25±0.3</td>
</tr>
<tr>
<td></td>
<td>3/6±0.4</td>
<td>0±1±0.1</td>
<td>3±0.4</td>
</tr>
<tr>
<td></td>
<td>4/1±0.5</td>
<td>0±15±0.1</td>
<td>3/5±0.2</td>
</tr>
<tr>
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<td>0±25±0.2</td>
<td>3/13±0.5</td>
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<tr>
<td>SFAS</td>
<td>6/5±0.5</td>
<td>0±2±0.1</td>
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<tr>
<td></td>
<td>6/5±0.7</td>
<td>0±23±0.2</td>
<td>3±0.4</td>
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<td></td>
<td>6/4±0.6</td>
<td>0±19±0.1</td>
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Momounsaturated fatty acid (MUFA)

<table>
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<tr>
<th></th>
<th>14:In-5</th>
<th>16:In-7</th>
<th>18:In-9</th>
<th>19:In-9</th>
<th>20:In-9</th>
<th>Total</th>
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<tr>
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<td>0/7±0.2</td>
<td>0/7±0.2</td>
<td>12±3</td>
<td>2/0±3</td>
<td>0/5±0.2</td>
<td>15±3±c</td>
</tr>
<tr>
<td></td>
<td>0/1±0.1</td>
<td>0/8±0.3</td>
<td>12±4</td>
<td>0/9±0.3</td>
<td>0/6±0.3</td>
<td>14±3±b</td>
</tr>
<tr>
<td></td>
<td>0/4±0.2</td>
<td>0/6±0.3</td>
<td>12±1/4</td>
<td>1/4±0.5</td>
<td>0/6±0.3</td>
<td>15±3±b</td>
</tr>
<tr>
<td></td>
<td>0/21±0.2</td>
<td>0/7±0.4</td>
<td>12±05±5/2</td>
<td>1/16±0.4</td>
<td>0/29±0.2</td>
<td>15±3±b</td>
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<tr>
<td></td>
<td>0/38±0.2</td>
<td>0/36±0.2</td>
<td>11/56±5</td>
<td>0/53±0.2</td>
<td>0/32±0.2</td>
<td>13/15±d</td>
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<td></td>
<td>0/47±0.2</td>
<td>0/42±0.3</td>
<td>10/99±5</td>
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<td>0/34±0.1</td>
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<td>10±8±4/6</td>
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<td>0/3±0.2</td>
<td>12±6±j</td>
</tr>
<tr>
<td></td>
<td>0/5±0.4</td>
<td>0/35±0.2</td>
<td>10±7±0.3</td>
<td>0/73±0.3</td>
<td>0/3±0.2</td>
<td>12±6±j</td>
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Polyunsaturated fatty acids (PUFA)

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<tr>
<th></th>
<th>18:3n-3</th>
<th>18:3n-6</th>
<th>20:2n-6</th>
<th>20:4n-6</th>
<th>20:5n-3</th>
<th>22:5n-6</th>
<th>22:6n-3</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>18/25±6</td>
<td>18±5</td>
<td>18±4/5</td>
<td>18±5</td>
<td>0/98±0.2</td>
<td>0/88±0.2</td>
<td>2±0.5</td>
<td>27/4±e</td>
</tr>
<tr>
<td></td>
<td>19±6</td>
<td>19/4±5</td>
<td>19/4±5</td>
<td>0/2±0.3</td>
<td>0/87±0.3</td>
<td>2±0.5</td>
<td>25/58±b</td>
<td>26/63±b</td>
</tr>
<tr>
<td></td>
<td>19/38±0.5</td>
<td>18±4±4</td>
<td>19/38±0.5</td>
<td>0/2±0.3</td>
<td>0/87±0.3</td>
<td>2±0.5</td>
<td>25/58±b</td>
<td>26/63±b</td>
</tr>
<tr>
<td></td>
<td>18±4±4</td>
<td>18±5</td>
<td>18±5</td>
<td>0/2±0.3</td>
<td>0/87±0.3</td>
<td>2±0.5</td>
<td>25/58±b</td>
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Fig. 4: Growth of biomass at different levels of lighting (Lv et al., 2010)

For the wavelength, microalgae usually uses between 400-700 nm wavelength 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 ($\lambda$=630-665nm) and blue light ($\lambda$=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. 6 shows that the blue light alone is not effective for growth. 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. Since the initial blue light creates a large cell size and high potential for secondary divisions under red light as well as increases in final products (Kim et al., 2014).

![Fig. 5: The effect of different wavelengths on cell size (Wang et al., 2007)](image)

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

**Temperature**

One of the most important environmental factors affecting various aspects of growth and fatty acid composition for more 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, limit cell growth speed and therefore reduces the biomass production (Nishida and Murata, 1996). The optimal temperature for *Chlorella vulgaris* is about 30°C which the most biomass productivity has been achieved (Chinnasamy et al., 2009; Xu et al., 2006). Converti et al., 2009 is reported that *Chlorella vulgaris* growth rate at 35°C decreases 17% compared with 30°C. An excessive rise in temperature to 38 °C leads to an abrupt halt the growth of microalgae and cells die. With increasing temperature to 30 °C cell growth rate increases and then decreases with increasing temperature to 35°C (Cassidy, 2011).
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 51 Mcell/ml were achieved in 9 days of culture at 30 °C is shown in Fig 7.

![Fig 7: Growth of biomass at different temperatures (Dvoretsky et al., 2015)](image)

In another study after 7 days of culture, optimum temperature 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% (Converit et al., 2009) and also protein synthesis (Konopka and Brock, 1978). An increase in temperature of 20 to 30°C, increases the concentration of intracellular free amino acids from 840 to 1810 mg per 100 g dry weight, followed by reduction of protein and starch (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 open system or photobioreactor and culture system scale as big or small which there are several ways to apply it (Ugwu and Aoyagi, 2012). The benefits of aeration or mixing include preventing precipitation of microalgae, homogenization of culture in order to access all of the cells to light and food, avoid creating temperature classes as uniform temperature and facilitate the exchange of gases between the culture and the air. Proportional with the scale of culture, mixing may be done by manual shaking on a daily basis on the 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 has obtained with aeration intensity 200 ml/min of 54 mg/L (Ogawa and Aiba, 1981). Amin et al. (2012) obtained the maximum concentration of biomass 2.05 g/L in aeration intensity 3 cm³/s. Kim et al. (2014) were reached cellular fatty acid content of 11.07% based on dry weight with air flow 100 ml/min. Although aeration is an important factor in the growth of biomass especially at the starting of the formation of the first nucleus of the cell and severity of aeration or unbalanced aeration can lead to the death of the first nucleus of cells. But, so far the direct effect of aeration on growth and cellular lipid production rate has been less studied and is intended often 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 that primary of them include: 1) Harvesting/dewatering: since algal cultures are mainly grown in water is required process 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 tow of most common is Folch and Bligh and Dyer method. 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 is 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 its heat of combustion. Cao et al. (2013) obtained biodiesel production efficiency about 92% by 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. Different species of microalgae for this purpose have been studied and evaluated. Among them Chlorella vulgaris due to 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 a organic carbon source (mixotrophic culture), will result 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 vulgaris carbon source is glucose and its optimal concentration is obtained 20 g/L with the largest biomass production 2.24 g/L, and the maximum lipid productivity 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, 3.43 g/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 its describing and more accurate analysis requires further researches. The best physical condition in order to achieve maximum growth performance and lipid production was obtained 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.

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

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