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Original Research Article

An investigation amount of cell density, biomass, lipid and biodiesel production in *Chlorella vulgaris* microalgae under effect of different parameters

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Key words: biodiesel; biomass; *Chlorella vulgaris*; microalgae

Abstract

Background and purpose: *Nowadays, microalgae are considered as the third-generation technology for the production of biodiesels in nature.* Chlorella vulgaris *is one of the most widely used microalgae for commercial purposes. It is one of the best options for producing biodiesel from algae because of its abundance and flexibility in cultivation. The systems applied for the production of algae are facing high expenses. One way to decrease the cost is to obtain the optimal values for the various parameters of the culture medium for maximum growth in algae.*

Materials and methods: *Therefore, in this study, the effect of parameters such as salinity, temperature, light intensity, light exposure time and acidity on cell density, biomass, lipid and biodiesel production was investigated.*

Results: *As a result, the highest cell density of* Chlorella vulgaris *was observed at 26 ˚C, light intensity of 3500 lux, light exposure of 17 hours, salinity of 5 ppm and pH of 7.5. The highest production of biomass was at light intensity of 3000 lux, light exposure time of 14 hours, salinity of 5 ppm and pH of 9. The highest lipid production was observed at 26 °C, light intensity of 4200 lux, light exposure time of 18 hours, salinity of 11 ppm and pH of 8. The highest biodiesel production was observed at 26 °C, light intensity of 4200 lux, light exposure time of 18 hours, salinity of 11 ppm and pH of 8.*

Conclusions: *These types of algae, which maintain themselves against environmental and non-biological stresses, show high specimen ability and resistance to recombinant shapes.*

INTRODUCTION

Today there are various crises such as limited fossil fuel sustainability, environmental concerns, acid rain, global warming, overcrowding, economic growth, and rising consumption *(1–3)*. The access of developing countries to new types of energy sources is essential for their economic development. Given the limited fossil energy reserves and the increasing level of energy consumption in the world, it is not possible to rely on existing energy sources. Also, most countries in the world have realized the importance and role of different sources of renewable energy *(4,5)*. Extensive research is being done extensively on the development of exploitation of these resources. Biodiesel is one of the most promising alternatives to fossil fuels, has many advantages including high flash point, high fat and biodegradability and can be used in

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compression combustion engines without any modification *(6)*. At present, biofuels are often produced from oilseeds, edible oil residues and animal fats *(7,8)*. Among these, algal sources (algae and microalgae) seem to be very suitable for biofuel production *(9)*. Microalgae are aquatic organisms in the micrometer dimension, often ranging in size from one to 400 micrometers, one of the oldest forms of life on earth *(10)*. Microalgae are considered as the third-generation technology for the production of biodiesels in nature and are considered as a source of renewable biological energy production *(11)*. Biodiesel production requires the breaking down of the triglycerides of natural oils into simple components, which can be done by pyrolysis, micro emulsion, and transesterification. Among these methods, the transesterification method is more widely used in terms of simplicity. This process is similar to the hydrolysis process, except that it replaces alcohol with water *(2,12)*. Biodiesel together with glycerol is produced from the reaction of triglyceride with methanol in the presence of a catalyst *(13)*. Catalysts (acidic (H_2SO_4 and HCl) and alkaline (NaOH and KOH) play an important role in the efficiency and production of biodiesel *(14)*. In some cases, enzymes can also be used as catalysts, but they are unstable and involve significant production costs *(5)*. Microalgae, like plants, can produce organic compounds using light and carbon dioxide *(15)*. Moreover, microalgae are able to produce all year round and therefore, have a higher efficiency compared to plants. Determining the optimal growth conditions for microalgae may lead to increase in industrial use of *Chlorella vulgaris*. These microalgae are used in aquaculture, biological energy, and cosmetics, furthermore, it has been used for many commercial purposes *(16)*. Considering the abundance and flexibility of microalgae in culture conditions, algae is one of the best options for production of biodiesel based on microalgae *(17,18)*. Large scale systems are used to produce algae, however, algae production in these systems is expensive. One way to reduce this cost is to use industrial wastewater. However, if the objective is to use microalgae in food production or cosmetic industry, microorganisms must be grown in clean conditions far from environmental pollutants *(19)*. It is obvious that microalgae react to the conditions of their growth environment by changing their physiological conditions *(20)*. This change can be considered as a feature of biotechnology that helps microalgae vary their structure and growth in response to change in conditions to achieve higher production. There is a tendency to use culture environments with low expenses to increase algae production *(21,22)*. Jorjani et al. *(23)* tested the effect of different salinity densities on growth of genus *Chlorella*. According to the obtained results, an upward trend was observed in growth of this alga at both control and saline treatments on the 5th day after inoculation. The optimum growth and the highest degree of special growth rate were seen in 0.5 % salinity. There were significant differences between the growth of this alga in control and saline treatments at 0.5 and 1 % salinity. The ability of this alga in carotenoid production in treatment with 1% salinity at the 9th day after inoculation were significantly more than the other treatments; however, this difference was not significant for chlorophyll production. Daliry et al. *(24)* investigated the optimal conditions for growth of *Chlorella vulgaris*. 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 photo bioreactor. 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 0.5 $g{\cdot}L^{-1}$ respectively. Alkaline medium (pH 9 to 10), non-continuous illumination, 5 to 7 Klux and a 200 $mL{\cdot}min^{-1}$ aeration flow rate, were known to be the best physical conditions. The most vulgaris biomass amount produced was 3.43 $g{\cdot}L^{-1}$, and the best lipid productivity was measured 66.25 $mg{\cdot}L^{-1}{\cdot}day^{-1}$. Allaguvatova et al. *(16)* considered the conditions for optimal growth of microalgae *Chlorella vulgaris*, stimulates the growth of agricultural plants and suppress the development of pathogenic microorganisms. The most favorable conditions for algae growth were daylight, temperature 25 °C and rotation at the speed 100 rpm. The most effective culture medium was of the bold basal medium with the addition of vitamins thiamine, cyanocobalamin, and soil extract. This method may use for creation the biopesticide and growth stimulators on the basis *Chlorella* biomass. Chia et al. *(25)* compared the growth of microalgae *Chlorella vulgaris* under different culture conditions. The need for clean and low-cost algae production demands for investigations on algal physiological response under different growth conditions. The highest cell density was obtained in LC Oligo, while the lowest in Chu medium. Chlorophyll a, carbohydrate and protein concentrations and yield were highest in Chu and LC Oligo media. Lipid class analysis showed that hydrocarbons, sterol esters, free fatty acids, aliphatic alcohols, acetone mobile polar lipids and phospholipids concentrations and yields were highest in the Chu medium. Triglyceride and sterol concentrations were highest in the LC Oligo medium. The results suggested that for cost effective cultivation, LC Oligo medium is the best choice among those studied, as it saved the cost of buying vitamins and EDTA associated with the other growth media, while at the same time resulted in the best growth performance and biomass production. Microalgae known as third generation technology for biofuel in nature are used as a renewable bioenergy source. Microalgae and crop plants have common for the production of organic compounds by using sunlight and carbon dioxide. In addition, microalgae are capable of being reproducing in the whole year allowing the more product yield than those of plants. Therefore, microalgae are more favorable feedstock since they have some advantages such as photosynthetic efficiency, biomass productivity and oil content. Determination of optimal microalga growth conditions may lead to an increase in the industrial applications of *Chlorella vulgaris* by Deniz *(26)*. Many researchers have recommended the ability of *Chlorella vulgaris* to produce biodiesel such as higher productivity, higher fat content and higher fatty acids. The researchers also reported that the fat content of microalgae rich in *Chlorella vulgaris* oil can reach 50 to 60% of the total dry *(27)*. Extensive research has focused on biodiesel production (temperature and pressure above 240 °C and 8.1 MPa). Higher product efficiency, shorter reaction time and no soap formation are the advantages of the supercritical methanol method. Microalgae have been converted to liquid fuel in supercritical methanol environments and offer a variety of products *(28,29)*. Another study found that biodiesel produced from microalgae lipids was 35% higher than lamb fat *(30)*. Also, under operating conditions, 97% of neutral lipids from *Chlorella vulgaris* were extracted using methanol (10% V/V) as an auxiliary solvent, which is a significant number *(31)*. Studies in an optimal condition showed that the lipid extract of microalgae is richer *(32)*. Considering the fact that cultivation of *Chlorella vulgaris* microalgae for producing biodiesel requires economic considerations and the cost of biomass, lipid and biodiesel production is very important, in this research different physicochemical conditions and their effect on growth of microalgae is examined to achieve the best conditions for production of biomass, lipid and biodiesel.

MATERIALS AND METHODS

Preparing microalgae

First, microalgae prepared from Jahad-Daneshgah Mashhad University was cultured in general culture medium of BBM (Bold's busal medium) in one liter presterilized containers *(33)*. All the necessary materials used in preparation of culture medium were from Merck products. *Chlorella vulgaris* microalgae were exposed to fluorescent lamps with an intensity of 3000 lux at a temperature of 22 to 25 °C *(34)*. For aeration, aquarium pump of Aqua model was used. After 5 days, 100 cc of microalgae were transferred to prepared media in the photobioreactor. Environmental factors were controlled in the second stage of production in a vertical tube photobioreactor *(35)*. Factors and related levels are completely presented in Table 1.

Biomass rate

To measure biomass, 400 ml of algal solution was taken from the sample and filtered with reweighted membrane filter papers. Filter paper containing algal solution was dried in an electric oven at 80 °C for 4 hours. After drying, the filter paper was kept in a desiccator. After reaching the temperature of the environment, it was weighed with a digital scale. The difference showed the dried weight of the microalgae. Considering that, the weight difference is related to 400 ml of filtered algae, to calculate the dry weight of 1 ml of algae, the number 400 was considered as V in the following equation to calculate the dry weight in milligrams per milliliter *(36)*.

$$DW\left(\frac{mg}{ml}\right)=\frac{DWA-DWC}{V}\times 1000 \qquad (1)$$

The definition of relationship was DWA: Dry weight of filter paper and algae, DWC: Dry weight of filter paper before filtering algae, V: The volume of filtered algal solution, N: Algae density in cells per milliliter.

Lipid extraction

Chemical extraction methods include the use of traditional organic solvents (hexane, ether, Chloroform, etc.) using supercritical fluid (SC-CO_2) and extraction with Soxhlet extractor. In this study, extraction was performed using Soxhlet apparatus. To do so, 10 g of each dried biomass sample was gently homogenized by a mortal and lipid extraction was performed by Soxtec 2050 Soxhlet apparatus. Each cycle consisted of boiling for 25 minutes, lipid extraction for 40 minutes and solvent recovery for 15 minutes. All samples were extracted equally in three cycles. To extract, three common solvents of diethyl, hexane and n-pentane with different boiling temperature and high purity were used. To remove, microalgae residues, the extracted lipids were filtered through a 45 micrometer filter. Each step of drying and lipid extraction was performed with three replications and the results were reported as average. After determining the amount of lipid, the dried lipid was dissolved in 4 ml of isopropyl alcohol and the amount of triglyceride in the lipid was measured *(37)*.

Table 1. *Characteristics of environmental factors and research varia*

Culture medium	Period length	Salinity (ppm)	Temperature (Celsius)	pH	Lighting duration	Intensity light (lux)	CO_2 (%)
BBM	15	5-8-11-14-17-20	20-22-24-26-28-30	5-6-7-8-9	14-15-16-17-18	3000-3500-4000-4500-5000	15

Biodiesel Extraction

Tran's esterification of fatty acids was performed in flasks containing sulfuric acid as a catalyst with a molar ration of 1:40 methanol to oil extracted at 180 rpm for 5 hours. Two layers were then formed, the upper layer contained biodiesel and was separated by petroleum ether *(38)*. The following equation (2 and 3) was used to determine the percentage of lipid production *(39)*.

Percentage of lipid production =

$$\frac{\text{Weight of extracted oil}}{\text{Biomass dry weight}} \times 100 \qquad (2)$$

Percentage of biodisel production =

$$\frac{\text{Weight of extracted biodisel}}{\text{Lipid weight}} \times 100 \qquad (3)$$

Statistical analyses

In this research, the variables and their levels were imported in Design Expert software and by using optimal level section the number of variables to be measured was 39 samples. After the experiment the data obtained from cell density, biomass, lipid content and biodiesel production were calculated and the analysis of variance at significance levels of 1% and 5% was used. The graphs were produced by this software *(40,41)*.

RESULTS

Various parameters of cell density

The most suitable growth process and the highest number of microalgae cells were observed. Considering the effect of temperature on cell density of microalgae, by

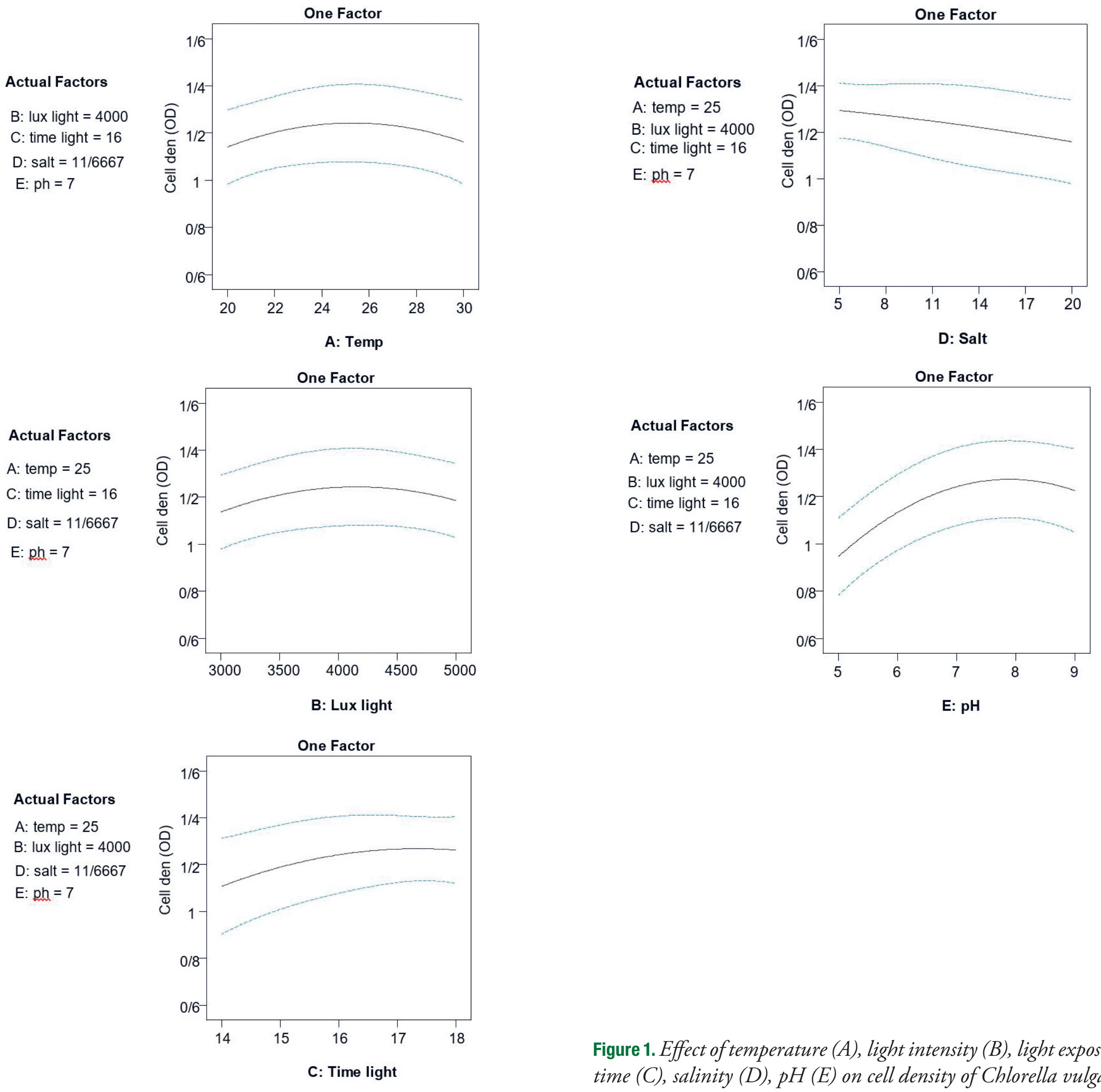

Figure 1. *Effect of temperature (A), light intensity (B), light exposure time (C), salinity (D), pH (E) on cell density of Chlorella vulgaris*

keeping constant values of light intensity at 4000 lux, pH at 7, salinity at 1 and light exposure time at 16 hours, results indicated that increase in temperature from 20 to 26 °C increases the cell density. The highest amount of cell density is observed at the temperature of 26 °C (Figure 1a). The amount of cell density increases from light intensity of 3000 to 3500 lux and after that, any increase in the light intensity decreases the cell density (Figure 1b). The best light intensity for cell density is 3500 lux. Regarding the effect of light exposure time on cell density by keeping other parameters constant, it is observed that by increasing the exposure time from 14 to 17 hours, cell density increases (Figure 1c). The highest cell density occurs at 17 hours of light exposure time. Environmental stress conditions reduce growth and weight in plants as well as algae. Regarding the effect of salinity in cell density, it is observed that with increasing salinity from 5 to 20 cell density decreases (Figure 1d). The highest cell density occurs in salinity 5 ppm. Increasing the pH from 5 to 7.5 leads to an increase in cell density (Figure 1e). The highest cell density is observed at pH 7.5.

Various parameters of biomass production

The best time to harvest microalgae under optimal growth conditions is two weeks after culture in a photobioreactor. Delayed growth of microalgae lasts for two days, after which logarithmic growth begins. Regarding the effect of temperature on the biomass, by keeping light intensity, light exposure time, salinity, and pH constant, it is observed that changes in temperature have no effect on produced biomass (Figure 2a). Regarding the effect of light intensity on biomass by keeping other parameters constant, it is observed that with increase in light inten-

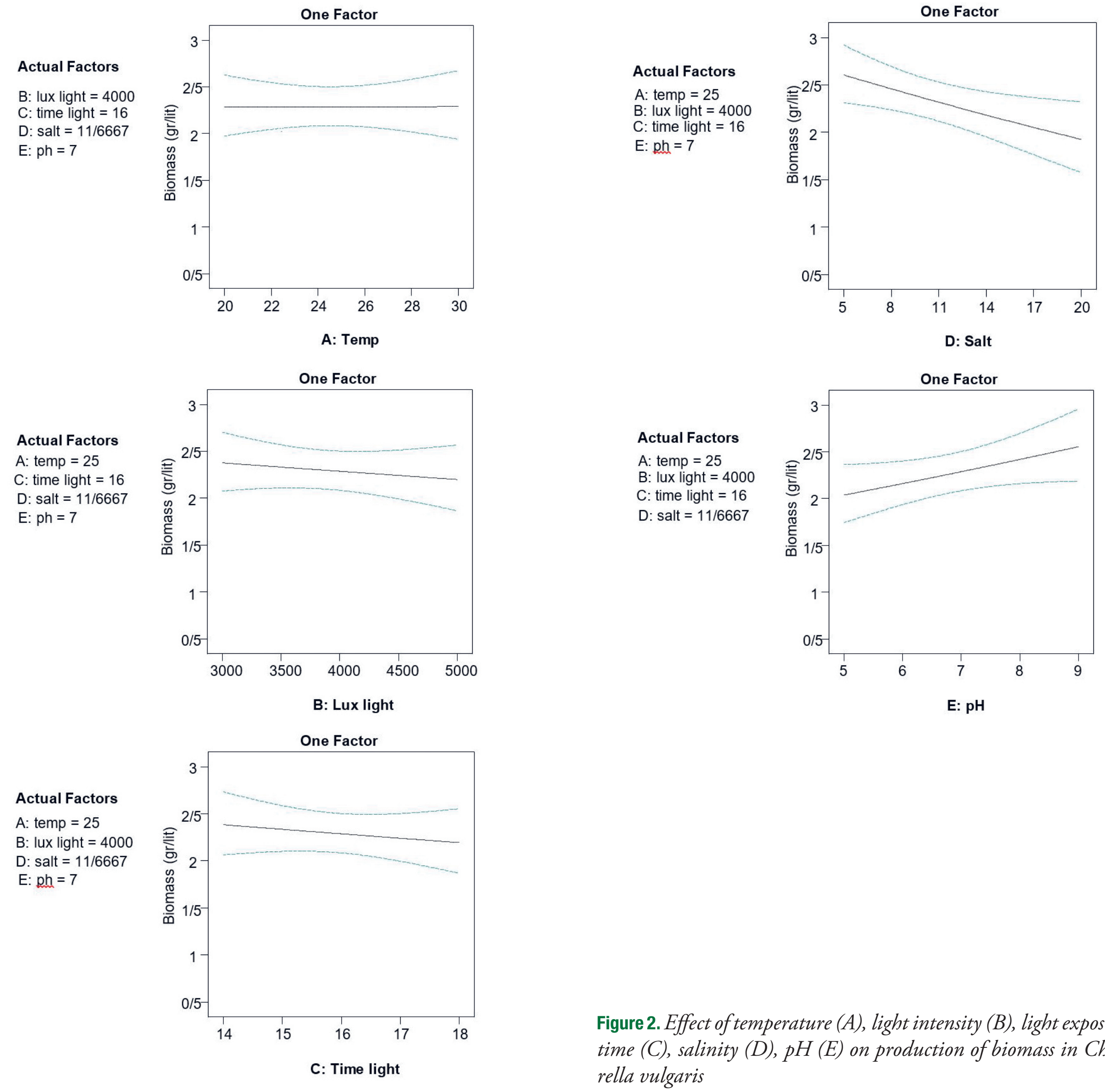

Figure 2. *Effect of temperature (A), light intensity (B), light exposure time (C), salinity (D), pH (E) on production of biomass in Chlorella vulgaris*

sity from 3000 lux, the amount of produced biomass decreases (Figure 2b). The highest amount of biomass is at light intensity of 3000 lux. Increasing the irradiation time from 14 hours leads to a decrease in the amount of biomass produced. Most biomass is produced in light exposure of 14 hours (Figure 2c). An increase in salinity from five reduces the amount of biomass produced (Figure 2d). Increasing the pH from 5 to 9 expands the amount of biomass produced (Figure 2e). The highest amount of biomass is observed at pH 9.

Various parameters of lipid production

Keeping light intensity constant, light exposure time, salinity, pH, and increasing temperature from 20 to 26 °C increased lipid production (Figure 3a). The highest amount of lipids is produced at 26 °C. Increasing the light intensity from 3000 to 4300 lux increases the amount of lipids produced. The highest amount of lipids is produced at 4200 lux light intensity (Figure 3b). Increasing the irradiation time from 14 to 18 hours increases the amount of lipids produced. The highest lipid production was observed at 18 hours of light exposure (Figure 3c). As salinity increased from 5 to 11, the amount of lipid produced increased. The highest amount of lipid was produced in salinity 11 (Figure 3d). Increasing the pH from 5 to 8 increases the amount of lipid produced and reaches a maximum at pH 8 (Figure 3e).

Various parameters of biodiesel production

Microalgae biodiesel has a higher viscosity and density than diesel fuel. Biodiesel has a lower calorific value than diesel fuel. Keeping other parameters constant along with increasing the temperature from 20 to 26 °C increases

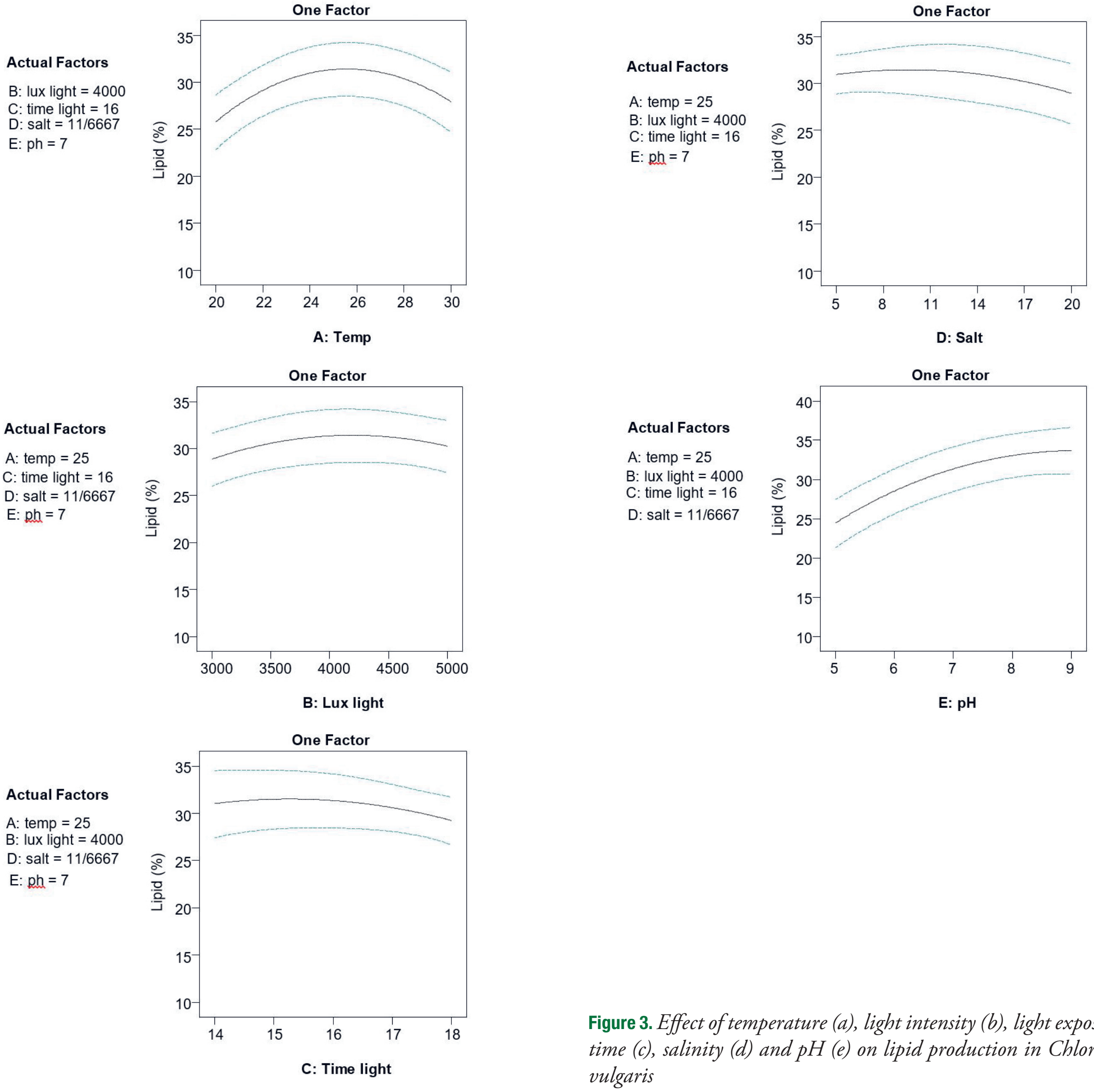

Figure 3. *Effect of temperature (a), light intensity (b), light exposure time (c), salinity (d) and pH (e) on lipid production in Chlorella vulgaris*

the amount of biodiesel produced. The highest rate of biodiesel production occurred at 26 °C (Figure 4a). Increasing the light intensity from 3000 to 4500 lux increases the amount of biodiesel produced. The highest amount of biodiesel is produced at a light intensity of 4500 lux (Figure 4b). Increasing the time of exposure to light slowly increases the production of biodiesel. Biodiesel production peaks at time light 18 (Figure 4c). Increasing salinity from 5 to 11 ppm slowly increases biodiesel production (Figure 4d). Increasing the pH from 5 to 8 increases the production of biodiesel and reaches a maximum in pH of 8 (Figure 4e).

DISCUSSION

Algae are now being considered as a new source of food, medicine, fuel, and so on. On the other hand, increasing salinity of water and soils as well as intense light are serious problems for the growth of algae and other living organisms. Sadeghi et al. *(23)* in a study on the effect of salinity on the growth of *Chlorella vulgaris*, results showed that the sharp increase in growth rate viewed at 5th day after inoculation at both control and treatments. Survivability would be kept at this salinity range. The optimum of growth and the highest degree of special growth rate were seen in 0.5% salinity. Difference between the growth at 0.5 and 1 % salinity were significant. The ability of carotenoid production at the 9th day after inoculation were significantly more than the other treatments and insignificant for chlorophyll production. In other study performed by Seyfabadi et al. *(42)* it was found that the highest growth rate of algae was at intensity of 100 micromoles of photons per square meter per second and light exposure time of 16 hours which was

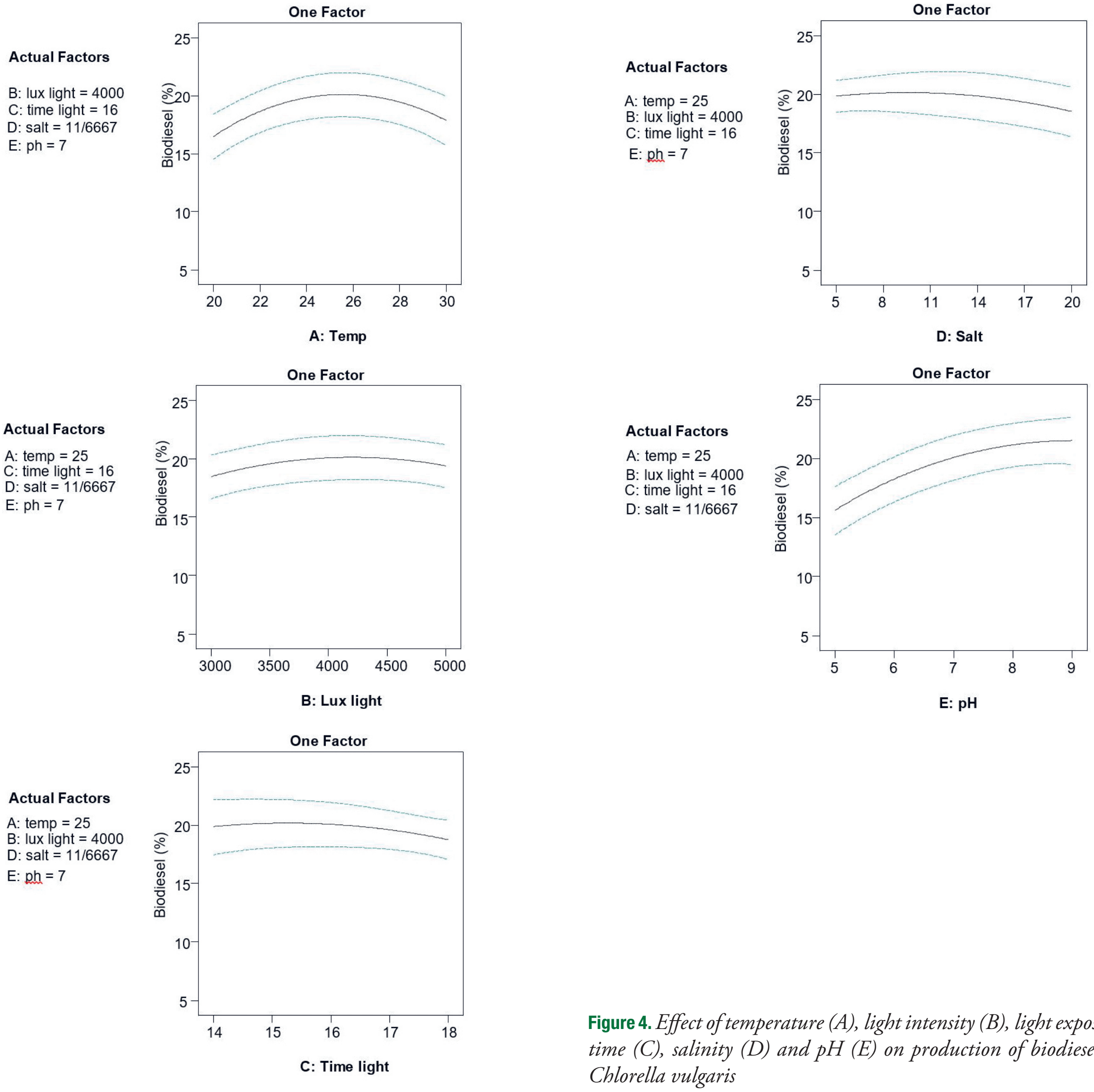

Figure 4. *Effect of temperature (A), light intensity (B), light exposure time (C), salinity (D) and pH (E) on production of biodiesel in Chlorella vulgaris*

close to the findings of this study. The highest biomass was produced at a light intensity of 62.5 micromoles of photons per square meter per second and a light exposure time of 16 hours, which was higher than findings of this study. Daliry et al. *(18)* showed that optimal growth of *Chlorella vulgaris*, glucose and nitrate levels should be 20 and 0.5 $g{\cdot}L^{-1}$, respectively. The culture medium should be alkaline (pH between 9 to 10). The exposure to light should not be continuous and the light intensity should be 5 to 7 thousand lux. The aeration rate should be 200 ml per minute. According to a report by Khalil et al. *(43)*, *Chlorella vulgaris* could grow in the pH range of 4 to 10, with the highest production occurring in an alkaline environment (pH 9 to 10). Yu et al. *(44)* showed that in autotrophic growth the acidity increases to 10, but in heterotrophic of mixotrophic growth, this number fluctuates around seven, which is close to the value obtained in this study. Gong et al. *(45)* found that the most suitable pH for the growth of *Chlorella vulgaris* is between 10 and 10.5. In another study lowering the pH had adverse effects on algal growth (Yan et al., 2013). He et al. *(46)* in study on the effect of physical factors on the growth of the *Chlorella vulgaris* found that the most suitable pH for the growth of algae is between 7 and 10. Khoeyi et al. *(47)* analyzed the light intensity, it was showed that exposure time of 16 hours versus 8 hours of darkness increased biomass production of *Chlorella vulgaris* and the best growth occurs in light intensity of 5000 lux. High light intensity such as 7000 lux decreases the biomass production. Other researches indicated that lipid production decreases with increase in light intensity *(48)*. In current research, lipid production decreased by increasing the intensity of light from 4200 lux. One of the most important environmental factors that affects various aspects of fatty acid growth and structure is temperature. Temperature can affect the reaction of enzymes, cell membrane systems and other properties *(27)*. Low temperatures limit cell growth rate and reduce biomass production *(49)*. In study by Converti et al. *(50)*, the optimum temperature for the growth of *Chlorella vulgaris* was 30 °C, at which the maximum biomass production was achieved. This temperature was higher than the temperature obtained for maximum production in this study. Converti et al. *(50)* found that the production rate of *Chlorella vulgaris* decreases by up to 17 % at 35 degrees, compared to 30 degrees. An increase in temperature to 38 degree leads to an immediate stop in the growth of microalgae and the cells die. In another study, the algal production increase to 30 °C and then decreases to 35 (Cassidy, 2011). Similar research has shown that the maximum biomass production rate is obtained at 30 plus and minus 2 °C and production decreases with increase in temperature to 35 plus and minus 2 *(51)*. Dvoretsky et al. *(50)* reported that the highest biomass production was achieved in 9 days at 30 °C. However, in this study, temperature changes had no effect on lipid production in the studied microalgae. Increasing the temperature from 25 to 30 °C of *Chlorella vulgaris* reduced the amount of lipids produced. In this research, the highest amount of lipid production was observed at temperature of 26 °C. In the study performed by Allaguvatova et al. *(16)*, the conditions for optimal growth of *Chlorella vulgaris* were under sunlight and temperature of 25 °C. On the optimal growth conditions of *Chlorella vulgaris*, temperature of 25 and pH of nine were measured as the best conditions for algae growth *(26)*. Although similar in temperature to the results obtained in this study, in terms of pH, higher values were suitable for maximum algae production in comparison with the results of this study.

CONCLUSION

A wide range of environmental and non-biological stresses such as high and low temperatures, drought and salinity can damage and be harmful to the plant. Salinity stress is one of the most important of them. Environmental factors that limit the growth and other activities of plants. Light as an energy source is an important factor for the life of plants and algae. Light also plays an important role in photosynthesis and accumulation of photosynthetic products. Various methods such as light inhibition affect algae and as a result are affected by stress. In terms of application, according to the present study, *Chlorella* microalgae were used as biofertilizer initiators and soil conditioners. Since it has the power to deal with salinity stress conditions, so it has a practical priority. Another important point is the specific issues of Iranian agriculture. Improper use of chemical fertilizers causes all kinds of destruction. These types of algae, which maintain themselves against these stresses, show high specimen ability and resistance to recombinant shapes.

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