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Optimization of Biodiesel Production Conditions Using *Chlorella vulgaris* Microalgae Cultivated in Different Culture Medium: Statistical Analysis

R. Farzandi[1], H. Nayebzaheh[2]*, M. Hokmabadi[1], N. Seghatoleslami[1]

[1] *Department of Chemical Engineering, Faculty of Engineering, Ferdowsi University of Mashhad, Mashhad, Iran*
[2] *Faculty of Material and Chemical Engineering, Esfarayen University of Technology, Esfarayen, North Khorasan, Iran*

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A B S T R A C T

The effect of cultivation culture on the biodiesel yield produced from in-situ transesterification of *Chlorella vulgaris* microalgae was assessed. Firstly, the algae were cultivated in Moh202, sterilized wastewater (SW), unsterilized wastewater (USW) mediums. It was found that around ten days were suitable to receive maximized growth of microalgae; while, maximum and minimum growth was detected in Moh202 and SW media. Before assessment, the effect of cultivation medium on the biodiesel content, the transesterification reaction conditions such as catalyst (NaOH) concentration, reaction time and amount of methanol were investigated by algae cultivated in Moh202 medium via fractional factorial design as statistical methodology. In the range of the study, catalyst concentration and reaction time were the most important effective parameters on the biodiesel yield. Moreover, the interaction between reaction time with catalyst concentration and amount of methanol was also important. In short reaction time and its interaction with catalyst concentration had positive effect, while catalyst concentration, amount of methanol and interaction of reaction time and amount of methanol had negative impact on the biodiesel yield. The yields of the algae cultivated in Moh202, sterilized and unsterilized wastewater media at the optimum conditions of 1 wt.% of catalyst, 9 mL methanol/g biomass and reaction time of 4 hours were 95.5%, 83.9% and 75.5%, respectively. Although the difference between biodiesel yields of *Chlorella vulgaris* Microalgae cultivated in the wastewater medium compared to sterilized wastewater medium was observed, wastewater can be used as a medium for cultivation of algae for biodiesel production to reduce the biodiesel production costs.

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INTRODUCTION

Widespread demand for energy due to population growth and industrialization leads to many problems such as environmental pollution and climate changes. Hence, there are many environmentally friendly solutions that renewable energy sources are one of them to reduce the energy demand and environmental crisis. Biodiesel as a renewable, biodegradable and clean fuel is considered as one of the best alternative fuels [1, 2]. Biodiesel is a mixture of fatty acid methyl esters (FAMEs), which are respectively produced by esterification, and transesterification of free fatty acids (FFAs) and triglyceride [3–5]. Biodiesel is mainly derived from renewable biological sources such as vegetable oils, animal fats which have disadvantages such as high cost and threat of human food supplies [6, 7].

Microalgae is well known for its rapid growth, short production cycle, high rate of oil production, and low competition or no competition with food production, which were studied in the biodiesel production processes [8–10]. *Chorella sp.*, *Dunaliella salina*, *Botryococcus braunii* and *Nannochloropsis sp.* are some of appropriate algae species used in biodiesel production processes due to their high lipids content [11, 12]. Depending on many parameters such as algae species and conditions of growth, the dry weight of algae can contain more than 80% lipids [13, 14]. The selection of algae species is important in determination of the rate of lipid production, which *Chlorella vulgaris* present high potential compared to other species [15, 16]. Talebi et al. [17] analyzed biomass productivity and lipid productivity as criteria for estimating the potential of different microalgae species which cultivated in Moh202 for production of biodiesel. They reported that *Chlorella vulgaris* had a high biomass (0.46 $gL^{-1}day^{-1}$) and volumetric lipid productivity (79.08 $mgL^{-1}day^{-1}$).

Another important parameter in the biodiesel production from microalgae is lipid extraction method, which is an essential step towards an economical

*Corresponding author E-mail: H.nayebzadeh@esfarayen.ac.ir (H. Nayebzaheh)

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biodiesel production [18–20]. Although physicochemical techniques such as ultrasonic blow-in, microwave, autoclave, bead-beating and sonication are commonly used for destruction of microalgae cell, this step is considered non-economical process [21–23]. Therefore, researchers focus on the direct or in-situ production of biodiesel from the microalgae, which unnecessary complex oil extraction stage will be eliminated. Tsigie et al. [24] reported that in-situ alkali catalyzed transesterification from dry algae biomass could result in a higher conversion (77.6%) in short time than when an acid catalyst was used. Nautiyal et al. [25] studied biodiesel production from algae species i.e. *Spirulina Chlorella* and pond water algae. These microalgae were cultivated in BG-11 medium and the oil was extracted to transesterification reaction. They also examined simultaneous extraction and transesterification using different solvents. Maximum biodiesel yield was obtained using hexane as a solvent. In addition, *Chlorella* Sp. had the highest growth rate and cell dry weight than the other two algae species. These researchers also concluded that biodiesel production efficiency in one step (oil extraction and simultaneous exchange of stearic) was higher than the two separate stages process.

One of the limitations of microalgae cultivation is the availability of food supplies at the industrial scale [26, 27]. A well-known, microbial culture system can play a valuable role in wastewater treatment, since microalgae are able to harvest and remove nutrients, heavy metals, organic matter and pathogens from wastewater [28]. Therefore, wastewater medium can be utilized as one of available and cost-effective medium for cultivation of microalgae [29, 30]. Feng et al. [31] were benefited of *Chlorella vulgaris* microalgae for treatment of sewage, reported the highest lipid (42%) and lipid efficiency (147 $mgL^{-1}d^{-1}$), while nutrients for micro-algae growth (COD and $+NH_4$) were removed from culture environment with 86% and 97%, respectively. Lim et al. [32] have shown that *C. vulgaris* was able to grow in textile wastewater (TW). The High rate algal pond (HRAP) system used in this study for bioremediation of TW remove up to 50% of color besides reducing pollutants such as COD, $NH_4 - N$ and $PO_4 - P$. Yuan et al. [33] assayed the cultivation of *Chlorella zofingiensis* in piggery wastewater for wastewater treatment and biodiesel production. Pollutants in autoclaved wastewater and NaClo-pretreated wastewater were utilized by *Chlorella zofingiensis* cultivated in doors. The FAME yield of *Chlorella zofingiensis* grown in autoclaved medium and NaClo-pretreated medium reached 10.18% and 10.15% of dry weight, respectively.

In this study, biodiesel production from *Chlorella vulgaris* algae cultivated in the three culture media via in-situ transesterification reaction (without extraction of oil) was investigated. *Chlorella vulgaris* microalgae were cultured in Moh202, sterilized and unsterilized wastewater media and the growth rate was evaluated. Then, the transesterification was performed to investigate the effect of cultivating medium on the biodiesel yield. Before that, the in-situ transesterification reaction conditions were optimized using full factorial design in two levels, which reaction time, amount of methanol and catalyst concentration were selected as independent variables and the yield was the response.

MATERIALS AND METHODS

Microalgae cultivation

Chlorella vulgaris microalgae were supplied from Karaj Biotechnology Center. At first, *Chlorella vulgaris* microalgae were cultivated in the Moh202 medium as a blank sample to compare the ability of other medium (sterilized wastewater (SW) and unsterilized wastewater (USW)) for cultivating *Chlorella vulgaris* microalgae. Moh202 medium contains the following compounds that are provided by Merck (Darmstadt, Germany):

1.25 g/L of $NaHCO_3$, 0.2 g/L of KH_2PO_4, 0.12 g/L of Vitamin B1, 0.1 g/L of KNO_3, K_2HPO_4, $MgSO_4$ and Vitamin B12, 0.03 g/L of $CaCl_2$ and NaCl , and 1 g/L Hunter's trace element. After adjusting the pH of medium and sterilizing at 121 °C for 15 min, the microalgae were mixed by Moh202 medium at volume ratio of 1:9. The oxygen of medium was provided by an aeration pump with a flow rate of 1.5 L/min after passing through a filter. The fluorescent light (3000 lux on a 16:8 hours light to dark cycle) was also used to supply constant light intensity for the algal medium and the temperature was set at 25±3 °C [15]. Figure S1 in the supplementary material depicts the cultivation medium.

Wastewater was collected from the entrance of Parkandabad wastewater's treatment plant in Mashhad and was clearly filtered to eliminate solid particles. It was used as a culture medium for algae growth. For this purpose, it was split to two parts such that one of them was utilized in the growing step without further treatment, while another part was sterilized as discussed in the above method (121 °C for 15 min).

20 mL of the blank sample cultivated in Moh202 was mixed with 200 mL of wastewater (and sodium bicarbonate as a carbon source was added. Then, the mixture was aerated and exposed under fluorescent light such as those cultivated in the Moh202 medium.

The growth rate of *Chlorella vulgaris* microalgae was daily measured by optical density measurement using spectrophotometry (UNICO2100, UV-VIS2100, USA) at 680 nm to assess the relationship between biomass concentration and optical density. After reaching the algal cells to the maximum growth, they were separated by centrifuging at 10000 rpm for 10 min and washed with distilled water to remove the remaining cells. Finally, these were dried in an oven at 45 °C for 24 h for further studies. Figure S2 in the supplementary material illustrates the dried microalgae.

In-situ transesterification reaction

The in-situ transesterification reaction was carried out in a 100 mL glass reactor connected to the condenser, which was poured by 10 g microalgae and desirable amount of catalyst (NaOH) and alcohol (methanol), which was determined using statistical analysis. The reactions were performed around the boiling point of methanol (65±3 °C), which was stabilized by an oil bath, for desirable duration. After completing the reaction, the mixture was discharged into the separation funnel and a small amount of heptane was added to facilitate the separation process. The upper layer containing biodiesel was separated from glycerol and carcass of microalgae layers. Then, it was washed twice by warm deionized water to eliminate the alkaline catalyst. Finally, the biodiesel was purified by heating to evaporate heptane, methanol and water. The biodiesel production process along with the obtained biodiesel from *Chlorella vulgaris* microalgae is illustrated in Figure S3 in the supplementary material.

The FAME compositions of the microalgae biodiesel were determined by gas chromatograph (GC, Agilent 7890A, USA) equipped with a mass spectrometer detector of the Agilent 5975C type and the HP-5 Mine Column (30m×0.25μm×0.25mm). Helium gas with a flow rate of 1 mL/min was used as carrier gas. The temperature of detector and injector were set at 250 °C [34].

Design of experiments

The experimental design was used for assessment the interaction of independent variables (reaction time (A), methanol amount (B) and catalyst concentration (C)) in the biodiesel production process from *Chlorella vulgaris* microalgae during in-situ transesterification reaction. The full-factorial design was utilized using the variables in two levels as listed in Table 1 to specify the amount of each material in the reaction and requirement reaction time. Moreover, two center points were selected in the middle range of all variables to fit the actual results with predicted equation, accurately. Design Expert software (version 6.0.2) was used to verify the data and expression of an appropriate equation, effect of interaction between independent variables on transesterification reaction and obtaining the optimum conditions to achieve the maximum efficiency.

TABLE 1. Independent variables (coded and actual values) of transesterification reaction

Variable	Factor code	Unit	Levels	
			Minimum	Maximum
Reaction time (t)	A	hour	2	4
Methanol amount (M)	B	mL/g biomass	9	12
Catalyst concentration (Cat.)	C	g/g biomass	1	3

RESULTS AND DISCUSSION

Algae growth rate

Duration of *Chlorella vulgaris* microalgae growth in three cultivation media was determined by examination of its growth pattern by daily measurements of optical density as shown in Figure 1(a). As well known, the growth of microalgae is generally characterized in five stages [35]. The first stage as called lag or induction phase is attributed to the physiological adaptation of the cell metabolism to growth that was two days for USW and Moh202 culture media and three days for SW culture medium. In the second stage, the growth of microalgae started as the plot shows a slope between second/third day and tenth day for Moh202 culture medium and ninth day for SW and USW culture media that is related to increase of concentration of microalgae in the culture medium. At the end of exponential phase, the growth rate reduced due to reduction the nutrients, light, pH, carbon dioxide or other physical and chemical factors caused to limit the growth [36]. This stage lasted two days for Moh202 and USW culture mediums and three days for SW culture medium. After receiving to the maximum growth, the growth maintained constant as called stationary phase (It was measured only for Moh202 culture medium that was three days). In this stage the limiting factors and the growth rate are balanced, which results in a relatively constant cell density. Reduction in the optical density was observed in fifth stage that is corresponded to decreasing the nutrients, as well as increasing the concentration of microalgae and the lack of light penetration [37]. Therefore, it seems that eleven days are appropriate for Moh202 and USW culture mediums and twelve days for SW culture medium to obtain the maximum microalgae growth.

Growth aspect of algal population has often been defined by specific growth rate. The specific growth rate of *Chlorella vulgaris* microalgae can be determined according to Equation (1) [38]. As well-known, this should be calculated only in the exponential phase of growth.

$$\mu = \frac{\ln(N(t)/N(0))}{t-t_0} \quad (1)$$

where μ is the specific growth rate, N(t) and N(0) are algae density (cell L^{-1}) at the time t and t=0, respectively and t is the cultivation time.

The specific growth rate of *Chlorella vulgaris* microalgae is shown in Figure 1(b). It observes that the specific growth rate is in the range of 0.15-.60, 0.23-0.56 and 0.29-0.77 for Moh202, USW and SW culture mediums, respectively. The growth rate in the USW culture medium is the lowest that can be related to existence of undesirable components in the culture medium, the high levels of toxics and the low light availability arising from self-shading at high algal density and other particles present in USW, which can affect the growth of microalgae [39]. On the other hands,

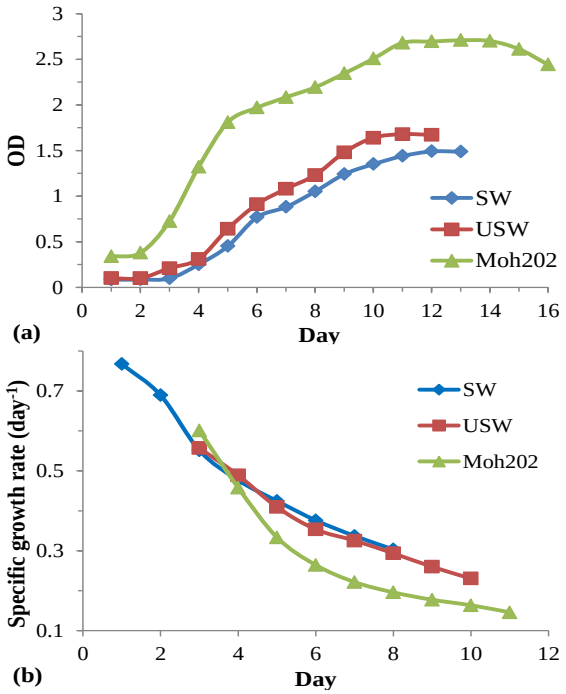


Figure 1. (a) Growth pattern and (b) specific growth rate plots of *Chlorella vulgaris* microalgae vs time in the three culture mediums

the specific growth rate of microalgae was significantly increased by cultivating the microalgae in the SW culture medium, because of appropriate availability of nutrients [16, 40].

Statistical assessment of the transesterification reaction conditions

The experimental design matrix and the yield of produced biodiesel from *Chlorella vulgaris* microalgae cultivated in Moh202 medium are presented in Table 2. The results revealed that the highest conversion (90.7%) was obtained at the conditions of 2 h, methanol-to-biomass of 12 mL/g and 1 wt.% of catalyst.

The effective factors on the biodiesel production were firstly selected to analyze the results and obtain an appropriate model. Figure 2 shows the normal distribution diagram of the effect of each factor. In fact, the effect of a factor which is far from the line is more important and has a greater impact on the response. The AB factor was the variable that had the greatest impact on biodiesel production efficiency. Subsequently, the factors C (catalyst), A (reaction time) and their interaction (AC) presented the most important parameters that use in the model for prediction of biodiesel yield. However, B, BC and ABC factors are invariant factors. On the other hands, due to influence of AB parameter on the model, B parameter was set in the model to reduce the error rate of the methanol parameter.

Table 3 shows the ANOVA analysis, which determines the significance of the parameters in relation

TABLE 2. Experimental design matrix and the responses

Run	Catalyst amount (wt.%)		Methanol/algae (mL/g)		Reaction time (h)		FAME (%)
	Actual	Coded	Actual	Coded	Actual	Coded	
1	1	-1	12	+1	2	-1	90.7
2	1	-1	9	-1	4	+1	95.5
3	3	+1	9	-1	4	+1	83.3
4	1	-1	12	+1	4	+1	78.9
5	3	+1	9	-1	2	-1	55.3
6	1	-1	9	-1	2	-1	73.5
7	3	+1	12	+1	4	+1	66.3
8	3	+1	12	+1	2	-1	70.5
9	2	0	10.5	0	3	0	75.2
10	2	0	10.5	0	3	0	77.3

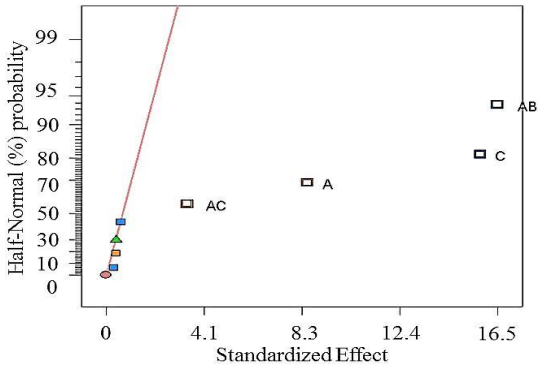


Figure 2. Distribution of the normal probabilities of each factor related to the percentage of conversion

to the values of p-value. As concluded from the result of Figure 2, A and C factors have major effects on the response due to their low P-value (lower than 0.05) that are important at 95% probability as well as the interactions of AB and AC. The curvature F-value as measured by difference between the average of the center points and the average of the factorial points in the design space is not significant. It confirms a linear model is sufficient to describe the response. Moreover, the lack of fit F-value is not significant relative to the pure error that is good for proposed model. As a result, the following equation according to coded values was presented:

$$Y = 73.76 + 24.4A - 16\,B - 90.7C - 26.8AB + 71.1AC \quad (2)$$

According to the suggested model, the amount of methanol, catalyst concentration and interaction of the reaction time and amount of methanol have negative effect on then response (biodiesel production). On the other hands, the reaction time and interaction of the reaction time and catalyst concentration have positive effect. According to coefficients of the parameters in the model, the reaction time and then the interaction of

TABLE 3. Analysis of variance (ANOVA) of suggested model

Variable	Sum of squares	Degree of freedom	Mean squares	F-value	F<prob.	
Model	1213.0	5	242.6	220.59	0.0005	Sig.
A	144.08	1	144.08	131.0	0.0014	Sig.
B	0.21	1	0.21	0.20	0.6886	not Sig.
C	499.44	1	499.44	454.12	0.0002	Sig.
AB	545.99	1	545.99	496.44	0.0002	Sig.
AC	23.29	1	23.29	21.18	0.0193	Sig.
Curvature	0.44	1	0.44	0.40	0.5707	not sig.
Residue	3.30	3	1.10			
lack of fit	1.12	2	0.56	0.26	0.8136	not sig.
Pure error	2.18	1	2.18			
Core total	1216.75	9				

reaction time and catalyst concentration have the highest effect. The accuracy of the suggested model can be verified by R^2, Adj. R^2 and Pred. R^2, where they were 0.9973, 0.9928, and 0.9782, respectively. The Pred. R^2 is in reasonable agreement with the Adj R^2 which indicates that the model is sufficient to express the relation between the response and the parameters. The results can be proved by Figure 3 which indicates the accuracy of predicted and actual values. The data obtained from predicted model has a good agreement with the experimental data.

Effect of the independent factors on the conversion of microalgae to biodiesel

The effect of the independent parameters on the biodiesel production from *Chlorella vulgaris* microalgae cultivated in the Moh202 medium is shown in Figure 4. In order to assessment each variable, other parameters were set at the center point. The effect of the reaction time on the yield (Figure 4(a)) presented that the higher conversion can be obtained at the higher reaction time because of the relevant time for reaction the reactants [2, 41]. Figure 4(b) shows the effect of methanol concentration on conversion rate. As shown in the model, methanol has no effect on the biodiesel efficiency. Although the amount of methanol has positive effect on the transesterification reaction [42]. Probably due to the use of raw algae and the choice of methanol based on the weight of algae, a higher amount of methanol than the proposed state has been used relative to the weight of oil present in algae. For this reason, methanol amount presented insignificant impact on biodiesel yield.

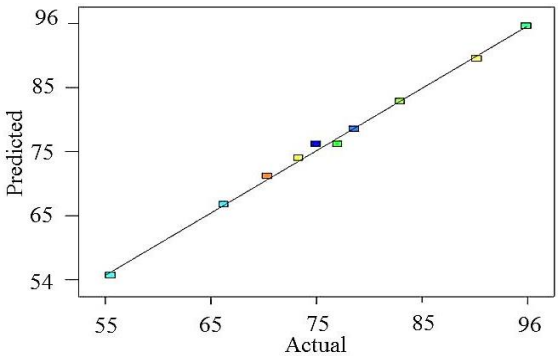


Figure 3. The fitting diagram of the actual data and the data obtained by the proposed equation

Figure 4(c) illustrates the effect of the catalyst on the biodiesel yield and confirms its negative effect, which means that the conversion rate decreases with increasing catalyst concentration in the reaction medium. The high amount of catalyst increases the saponification reaction rate and prevents the conversion of FFA and triglycerides to biodiesel [43]. Therefore, the reaction yield will be decreased.

Effect of the interaction of parameters on the conversion of microalgae

The interaction of the reaction time with the amount of methanol and catalyst concentration, while another parameter is constant at the central point) is shown in Figure 5 as a three-dimensional plot. The interaction effect of the amount of methanol and reaction time, as shown in Figure 5(a), presented that although the yield increased by increasing each parameter at the lowest level of other parameter, the oppositional behavior observes at the highest level of each parameter. In other words, after the center point for the both variables, with increasing of each of the parameters at constant amount of other parameter, a reduction in the biodiesel production yield observes. This can be due to the fact that with increasing reaction time, due to receiving to the equilibrium point, the reaction goes in the backward direction and the excessive increase in methanol causes problems in the separation of the glycerol phase from the biodiesel, which can affect the efficiency [2].

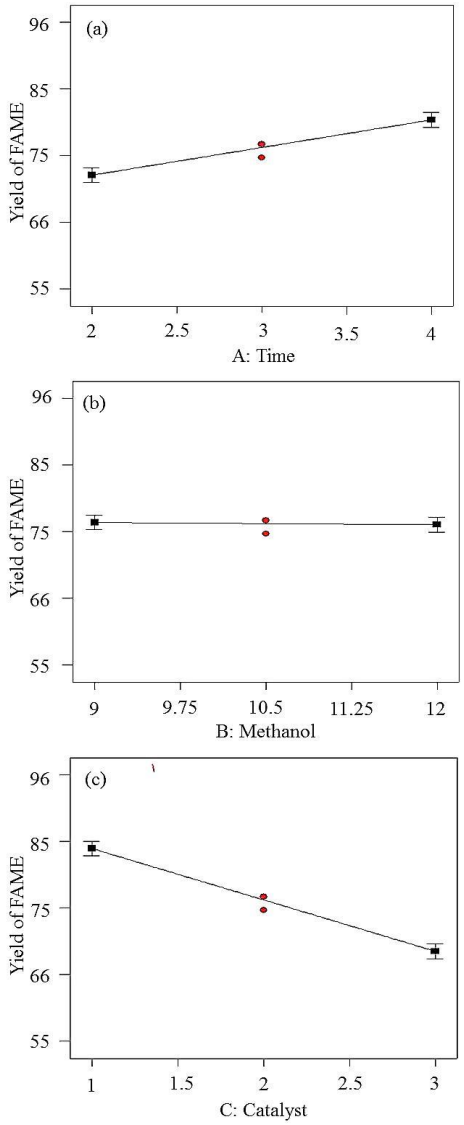


Figure 4. The effect of (a) reaction time, (b) methanol amount and (c) catalyst concentration as the independent variables on the biodiesel yield

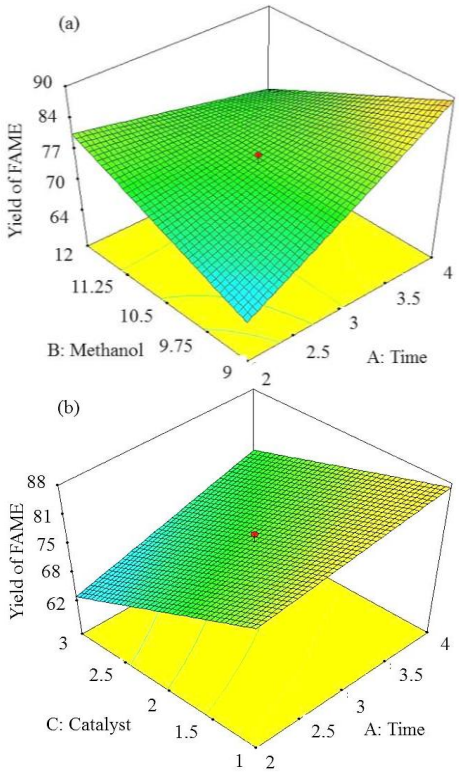


Figure 5. Three-dimensional plots of the influence of (a) reaction time and methanol amount and (b) reaction time and catalyst concentration as interaction parameters on the biodiesel production

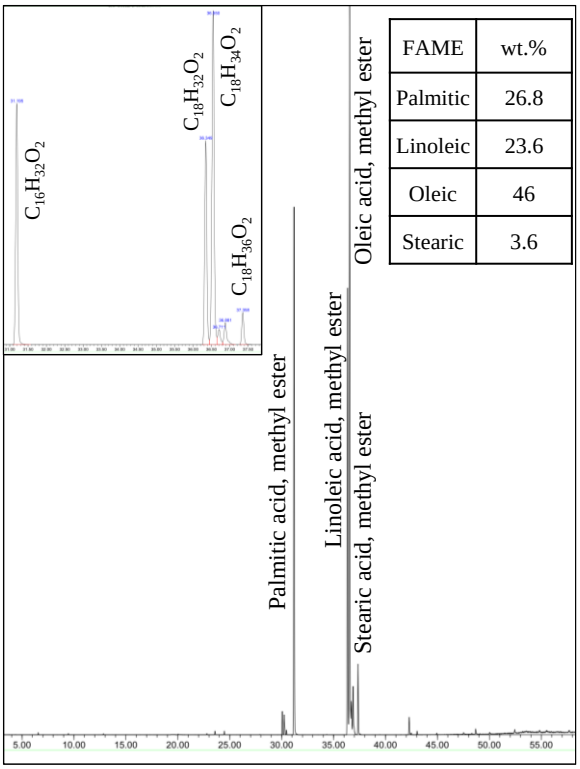
Figure 5(b) shows the interaction between the catalyst concentration and reaction time at the methanol amount of 10.5 mL/g biomass. The negative effect of increase the catalyst concentration observes in all the range of reaction time while the reaction time shows the positive influence in the all range of catalyst concentration. Therefore, the highest reaction time and lowest catalyst concentration results the highest yield.

Optimization the in-situ transesterification reaction conditions

Optimization the reaction conditions of in-situ transesterification of *Chlorella vulgaris* microalgae was performed by Design expert 6.0.2 software. After evaluation the suggested conditions to obtain the maximum FAME content based on selecting the other parameters in the examined range, the operation conditions of 1 wt.% of catalyst, the methanol-to biomass ratio of 9 (mL/g) and the reaction time of 3.99 h were chosen. The biodiesel yield of 95.49% was reported, which is in good agreement with the experiments value (95.5%). The GC-Mass plot and compositions of the produced biodiesel are depicted in Figure 6. The produced biodiesel contained oleic, palmitic, linoleic, and stearic acids as the major components.

Assessment the properties of microalgae cultivation medium on the FAME content

Chlorella vulgaris microalgae cultivated in the non-sterile and sterile wastewater medium were dried in oven and used in the in-situ transesterification reaction at the optimum conditions. The FAME content of the produced biodiesel from *Chlorella vulgaris* microalgae cultivated in three cultivation medium is illustrated in Figure 7. The FAME content is higher for microalgae cultivated in the SW medium which could be due to this fact that SW medium has the most growth associated with the *Chlorella vulgaris* algae species, since this type of algae has grown better in sterile conditions. However, under USW conditions, the lipid content may have decreased, where other species can grow in addition to *Chlorella*



FAME	wt.%
Palmitic	26.8
Linoleic	23.6
Oleic	46
Stearic	3.6

Figure 1. GC-Mass plot of produced biodiesel and fatty acid methyl ester (FAME) profile

algae type, leading to not-well growth of *Chlorella sp.* algae. Moreover, the biodiesel production efficiency has decreased in USW medium. It must be mentioned that there are other bacteria in the wastewater which grown simultaneously with algae, so the biomass concentration is sum of algal and any survival bacteria in the wastewater. Therefore, less amount of microalgae are presented in the mixture and these bacteria can eliminated the well-growth of microalgae [44].

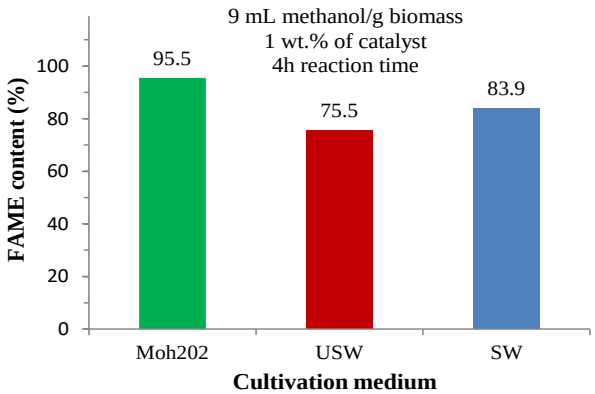


Figure 7. FAME content of produced biodiesel from *Chlorella vulgaris* microalgae cultivated in three different mediums

The results of this study were in accordance with the results reported by Li et al. (2011). The FAME yield in autoclaved wastewater is similar to that in tris-acetate-phosphate (TAP) media. The most abundant fatty acids obtained from algae were also octadecadienoic acid (C18:2) and hexadecanoic acid (C16:0). Different from algae cultivated in TAP media and autoclaved wastewater, octadecatrienoic acid (C18:3) (18.79% of total FAME) and hexadecanoic acid (C16:0) (16.10% of total FAME) accounted for the majority of the fatty acids for algae cultivated in raw wastewater. Finally, based on biodiesel production efficiency using the SW medium, it can be concluded that wastewater can be used as a medium for algae growing and biodiesel production, associating with significant decreasing in the biodiesel production costs.

CONCLUSION

In this study, the amount of produced biodiesel from oils of *Chlorella vulgaris* microalgae cultivated in the three cultivation medium was studied during in-situ transesterification reaction. The Moh202, USW and SW medium were used for cultivating the microalgae. The transesterification reaction conditions were firstly optimized by the microalgae cultivated in the Moh202 medium where three variables including reaction time, methanol amount and catalyst concentration were considered as independent parameters. The full factorial design was utilized to accurately evaluate the variables and the effect of their interactions on the FAME content of produced biodiesel. The results showed that the catalyst concentration, reaction time, and interaction of reaction time with methanol and catalyst amount have a significant effect on the conversion of microalgae. The proposed model showed excellent accuracy for prediction the conversion of microalgae in the in-situ transesterification reaction and the optimum conditions of 1 wt.% of catalyst, 9 mL methanol per gram of biomass, and the duration of 4 h was obtained. Cultivating of microalgae in the SW and USW mediums showed that the specific growth rate of microalgae in the SW in higher than other medium and the produced biodiesel from the microalgae cultivated in this medium has higher FAME content compared to those cultivated in the USW medium. Although the yield of biodiesel produced from microalgae cultivated in SW medium was slightly lower than those cultivated in the Moh202 medium. This medium can be utilized as high potential and cost-effective medium for reduction the cost of biodiesel production process. Moreover, it must be mentioned that microalgae have high ability for removing of wastewater pollutants which is an important environmental issue.

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Persian Abstract

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چکیده

اثر محیط کشت بر عملکرد بیودیزل تولید شده از ترانس استریفیکاسیون از ریز جلبک‌های *کلرلا ولگاریس* ارزیابی شد. در مرحله اول، جلبک‌ها در محیط‌های Moh202، فاضلاب استریلیزه (SW)، فاضلاب استریل نشده (USW) کشت شدند. مشخص شد که حدود ده روز برای دریافت حداکثر رشد ریز جلبک مناسب است. در حالیکه، حداکثر و حداقل رشد در محیط Moh202 و SW مشاهده شد. قبل از ارزیابی، اثر محیط کشت بر روی محتوای بیودیزل، شرایط واکنش ترانس استریشن مانند غلظت کاتالیزور، زمان واکنش و مقدار متانول توسط جلبک‌های کشت شده در محیط Moh202 از طریق طراحی فاکتوریل کسری به عنوان روش آماری بررسی شد. در دامنه مطالعه، غلظت کاتالیزور و زمان واکنش مهمترین پارامترهای موثر بر عملکرد بیودیزل بودند. علاوه بر این، تعامل بین زمان واکنش با غلظت کاتالیزور و مقدار متانول نیز مهم بود. در زمان واکنش کوتاه و اثر متقابل آن با غلظت کاتالیزور اثر مثبت داشت، در حالی که غلظت کاتالیزور، مقدار متانول و اثر متقابل زمان واکنش و میزان متانول بر عملکرد بیودیزل تأثیر منفی داشت. بازده جلبک‌های کشت شده در Moh202، محیط فاضلاب استریلیزه و غیر استریل شده در شرایط مطلوب ۱ درصد وزنی کاتالیزور، ۹ میلی لیتر متانول در گرم زیست توده و زمان واکنش ۴ ساعت به ترتیب ۹۵٪/۵، ۹٪/۸۳ و ۷۵/۵٪ بود. اگرچه تفاوت بین عملکرد بیودیزل ریز جلبک‌های *کلرلا ولگاریس* کشت شده در محیط فاضلاب در مقایسه با محیط فاضلاب استریلیزه مشاهده شد، می‌توان از فاضلاب به عنوان محیط کشت جلبک برای تولید بیودیزل استفاده کرد تا هزینه‌های تولید بیودیزل کاهش یابد.