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Optimization of some fermentation conditions for bioethanol production from microalgae using response surface method

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Abstract

Background: Algal biomass fermentation is one of the promising alternatives for bioethanol production. The bioethanol yield relies on fermentation conditions as the algal biomass amount, the yeast volume (% v/v), and the fermentation time. In this work, algal biomass harvested from a pilot-scale high rate algal pond (HRAP) was fermented anaerobically using immobilized *Saccharomyces cerevisiae* (ATCC 4126). The HRAP was constructed at the Zenin wastewater treatment plant (WTP), Giza, Egypt. A separate hydrolysis fermentation process (SHF) was applied for algal biomass. The effect of the algal biomass amount, the yeast volume (% v/v), and the time of fermentation as three independent variables were studied simultaneously and analyzed statistically using Design-Expert software V6.0.8.

Results: The harvested algal biomass from HRAP contains 45% carbohydrates and was dominated by *Microcystis* sp. The results revealed that optimum bioethanol yield 18.57 g/L is achieved by fermenting 98.7 g/L algae using 15.09% of the volume immobilized yeast for 43.6 h with a 95% confidence interval.

Conclusion: Microalgae grown on wastewater are a promising source of bioethanol production. Maximizing the ethanol production is achieved by optimizing the fermentation parameters as algal biomass, fermentation time, and yeast volume percent. The simultaneous optimization of the parameters using a statistical program is an effective way to maximize the production and predict a model that describes the relationship between these parameters and their response. The prospective research is going to study the effect of these predicted parameters on continuous fermentation on the semi-pilot scale.

Keywords: High rate algal pond, Bioethanol, *Saccharomyces cerevisiae*, Immobilized yeast, Fermentation time

Background

The concern for the deleterious effect of fossil fuels on the environment obliges the society to reduce greenhouse gas emissions via creating renewable fuel alternatives (Demirbas 2009, Wolske and Stern 2018, Jorgenson et al. 2019). Among renewable energies, the preference was given to liquid biofuels as it represents about 40% of the total energy consumption in the world (Tan et al. 2008). The global ethanol production has been altitude from 13.12 billion gallons in 2007 to 27.05 billion gallons in 2017 with a slight reduction in 2012 and 2013 (Renewable Fuels Association US 2018). Bioethanol can be used as it is or blended with gasoline to form "gasohol" (Staniszewski et al. 2007). Also, it can be used as a gasoline improver or octane enhancer and in bioethanol-diesel blends to reduce the emission of exhaust gasses (Pejin et al. 2009). In contrast to petroleum fuel, bioethanol is readily biodegradable, less toxic, and emits lesser airborne pollutants (John et al. 2011).

Algal growing in wastewater can significantly share in the management of freshwater ecosystems and treat wastewater. The integrated algal system has other advantages than wastewater treatment depending on the algal community as a source of biofuel production. Microalgae are a potential feedstock for bioethanol production, as they possess high concentrations of carbohydrates (11–50%) in the form of starch and cellulose, which can be fermented to bioethanol (Lu et al. 2001, Yusuf 2007, Silva and Bertucco 2016, Doma et al. 2018, Silva et al. 2018). Microalgal carbohydrates lack lignin, which makes

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their conversion to monosaccharides much easier compared to lignocellulosic materials (John et al. 2011).

Bioethanol can be produced from micro-algal biomass via a fermentation process by *Saccharomyces cerevisiae* (Nigam and Singh 2011), the common microbe used in ethanol production according to its ability to ferment a wide range of sugars, its high ethanol tolerance, and its high ethanol productivity (Kasavi et al. 2012, Lin et al. 2012). The efficiency and productivity of ethanol can be enhanced by immobilizing the yeast cells (Jin and Speers 1998, Domingues et al. 2000). It reduces the cost of cell recovery as it separates easily from the fermentation medium without centrifugation (Choi et al. 2010). Production of bioethanol during fermentation depends on several factors such as inoculum size, sugar concentration, agitation rate, temperature, pH, and fermentation time (Attfield 1997), Tofighi et al. 2014).

In this work, three independent parameters algal biomass, percent yeast volume, and fermentation time which significantly affect the fermentation yield were investigated. A model that describes the bioethanol production as a function of the studied parameters was created. Most prior research has studied these parameters separately, simply one of the three parameters was invariably kept constant while varying the other, thus hindering the opportunity of any potential interaction between the three factors. In this respect, designing experiments using response surface methodology (RSM) allows studying the simultaneous influence of several parameters through statistical methods. One of the merits of that system is the possibility of determining any nonlinear relationships between the independent variables (Montgomery 2003). This step was enhanced by establishing a model for predicting the bioethanol production.

Material and methods

The algal biomass production system

An integrated system was constructed at the Zenin wastewater treatment plant (WTP), Egypt. This system consists of a primary facultative pond followed by the high rate algal pond (HRAP) with dimensions of 7.5 m × 2.4 m × 0.3 m (L × W × H) and active volume of 5.4 m^3. The inlet of the system was fed from Zenin WTP influent after physical treatment (screening) as illustrated in Fig. 1 followed by a facultative pond where the organic matter was decomposed by bacteria.

Algal biomass grew in HRAP along the operating period from June to December 2017 and was dominated by *Microcystis* sp. A microscopical investigation for the algal community was carried out three times a week. Harvesting was carried out through coagulation by cationic starch (10 mg/L) followed by settling for an hour (El-Naggar et al. 2018). The precipitated algae were collected and dried using a solar dryer. The dried algal biomass was hydrolyzed to digest the complex sugars preparing it for fermentation via immobilized *Saccharomyces cereviciae* (ATCC 4126).

Immobilized yeast preparation

The inoculum was prepared by transferring the *S. cerevisiae* cells into the yeast peptone dextrose broth media (YPD-Himedia, M1363) then incubated the cultures at 37 °C for 48 h. The yeast culture was centrifuged at 4000 rpm; the settled pellets were suspended in 0.9% (w/v) NaCl solution then centrifuged and immobilized in Na–alginate. The yeast cells were immobilized in Na–alginate as described by El-Dalatony et al. (2016). The 2% (w/v) Na–alginate solution was prepared by dissolving 7 g of Na–alginate powder (Alfa Aeser) into 360 mL of distilled water by gradual adding the powder into the bottle while gently steering. Ninety milliliters of yeast biomass suspension was gently added to the Na–alginate mixture and mixed thoroughly at room temperature. This mixture was extruded drop-wisely through a 50-mL burette to the $CaCl_2$ (2.65% w/v) solution to prepare uniform spherical beads (≈ 0.4 mm diameter). The resulting beads were then washed with distilled water

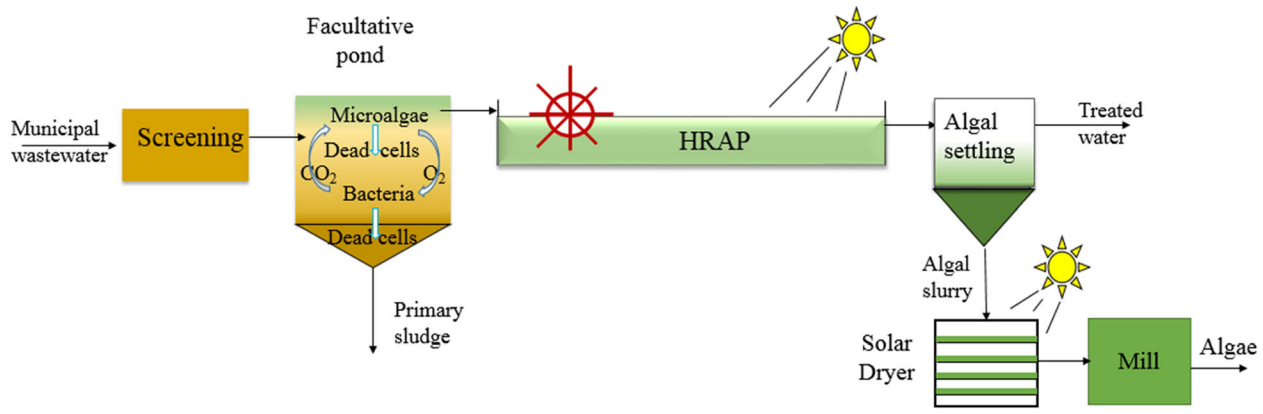

Fig 1 Schematic diagram of the HRAP system at Zenin WWTP

two to three times to remove the impurities and stored at 4 °C for 10 min for hardening.

Acid hydrolysate preparation of microalgal biomass

The feed stream was hydrolyzed using 0.5 N H_2SO_4 for 4 h at temperature 120 °C. The hydrolyzed biomass was subsequently adjusted to pH 4.5 (± 0.1) with NaOH at temperature 30 °C then filtrated. The carbohydrate concentration was measured for the hydrolysate by spectrophotometer (Cary 100 UV-Vis) at 485 nm (Dubois et al. 1956).

Fermentation

The hydrolysate of algal biomass was fermented anaerobically with immobilized *S. cerevisiae* at 30 ± 2 °C. The algal hydrolysate was covered with paraffin film to ensure an anaerobic condition. Fermentation processes were implemented in three parallel containers 500 mL each and operated at the same time to unify the conditions. Bioethanol produced was separated from the aqueous solution by evaporation at 70 °C using the rotary evaporator where the condensed bioethanol was collected under the ice to avoid re-evaporation (Khalil et al. 2015).

The ethanol purity was measured calorimetrically using the potassium dichromate method (Crowell and Ough 1979) by adding 2 mL of distilled sample to 10 mL of acidic potassium dichromate reagent and mixed well. The tubes were capped and kept in a water bath at 60 °C for 20 min then cooled to room temperature. The absorption of the reaction mixture was measured at 600 nm by spectrophotometer (Cary 100 UV-Vis). The potassium dichromate reagent was prepared by dissolving 34 g of potassium dichromate ($K_2Cr_2O_7$) in 400 mL distilled water with 325 mL of sulfuric acid and by making up the volume to 1 L. A standard curve was prepared under similar conditions using standard solutions of ethanol in distilled water (Khalil et al. 2015). The fermentation yield percent is calculated as follows (Silva et al. 2018):

$$\text{Fermentation yield}\% = [\text{Ethanol produced}/(0.511 \times \Delta\text{sugars})] \times 100 \quad (1)$$

Response surface methodology

The algal biomass, the incubation time of fermentation, and the volume ratio of yeast to the algal substrate are considered the most significant parameters affecting the bioethanol production (Attfield 1997, Choi et al. 2010, Tofighi et al. 2014). The range of the examined algal biomass was 30–100 g/L. The selected range of incubation time was 24–72 h. The volume ratio of immobilized yeast to the algal substrate was ranged from 1:10 to 1:1, i.e., yeast occupies a range of 15–50% of the total volume. The RSM was chosen as a suitable route for optimizing the interactive effect of algal biomass (G), the fermentation time (T), and the yeast volume percent (V) simultaneously on the bioethanol production.

The experimental results were statistically analyzed and modeled using the RSM via Design-Expert 6.0.8 software during a trial period. The extent of the fit of the model was evaluated using the normal plot of residuals and the analysis of variance (ANOVA).

Results

The effects of algal biomass (G), the fermentation time (T), and the yeast volume % v/v (V) were studied as the three independent variables affecting bioethanol production in a total of 20 experiments through a central composite design as shown in Table 1. The data were analyzed using Design-Expert 6.0.8 software. Table 2 describes the ANOVA for the response surface of a quadratic model for bioethanol response. The analysis of the response yielded 3 linear coefficients (G, V, T), three quadratic coefficients (G^2, V^2, T^2), and three cross-product coefficients for the full model ($G \times V$, $G \times T$, $V \times T$). The statistical analysis of the response revealed that the regression coefficient (R^2) was 0.998, whereas the predicted R^2 was 0.992 and the adjusted R^2 was 0.997 with a coefficient of variation (CV) of 1.5. The probability of F function for each model term is less

Table 1 Experimental data for central composite design

Run	Algal biomass, g/L	Yeast volume % v/v	Fermentation time, h	Bioethanol production, g/L	
				Actual	Predicted
1	65.00	3.07	48.00	18.53	18.49
2	100.00	50.00	24.00	13.82	13.88
3	30.00	15.00	72.00	13.04	12.98
4	65.00	32.50	88.36	13.49	13.32
5	6.14	32.50	48.00	3.92	4.04
6	65.00	32.50	7.64	10.98	11.15
7	65.00	32.50	48.00	14.17	14.20
8	65.00	32.50	48.00	14.05	14.20
9	65.00	32.50	48.00	14.20	14.20
10	65.00	32.50	48.00	14.50	14.20
11	123.86	32.50	48.00	15.04	14.92
12	100.00	15.00	24.00	18.01	17.90
13	30.00	15.00	24.00	8.00	8.00
14	100.00	15.00	72.00	17.76	18.00
15	30.00	50.00	72.00	8.33	8.44
16	65.00	32.50	48.00	14.15	14.20
17	65.00	61.93	48.00	11.24	11.28
18	65.00	32.50	48.00	14.10	14.20
19	100.00	50.00	72.00	11.46	11.46
20	30.00	50.00	24.00	6.21	5.97

Table 2 ANOVA results for the quadratic model

Source	DF	*F*-value	Prob > *F*
Model	9	831.63	< 0.0001
A	1	3882.78	< 0.0001
B	1	1702.62	< 0.0001
C	1	153.10	< 0.0001
A^2	1	1088.45	< 0.0001
B^2	1	23.30	0.0007
C^2	1	188.10	< 0.0001
AB	1	54.08	< 0.0001
AC	1	324.27	< 0.0001
BC	1	85.95	< 0.0001
Lack of fit	5	1.93	0.2446
R^2	0.998		
Adjusted R^2	0.997		
Predicted R^2	0.992		
C.V	1.5		

than 0.05 which verifies the significance of all the model terms, while the probability of *F* function for the lack of fit is greater than 0.05 which verifies the non-significance of error.

The regression equation of the bioethanol production response in terms of the actual variables is as follows:

$$\begin{aligned}&\text{Bioethanol production, g/L}\\&= -5.39 + (0.37 \times G) - (0.05 \times V)\\&\quad + (0.29 \times T) - \left(1.3 \times 10^{-3} \times G^2\right)\\&\quad + \left(8 \times 10^{-4} \times V^2\right) - \left(1.2 \times 10^{-4} \times T^2\right)\\&\quad - (0.003 \times G \times V) - (0.002 \times G \times T)\\&\quad + (0.003 \times V \times T)\end{aligned} \quad (2)$$

The predicted model is verified experimentally through the actual values of the bioethanol production and the equivalent predicted values as shown in Table 1. The optimum predicted result is 18.57 g/L that is shown in Table 3 and was verified experimentally through triplicate fermentation experiment for 65 g/L algal biomass, 32.5 yeast volume % v/v, and 43.6 h. The actual results were 18.54 g/L, 18.56 g/L, and 18.58 g/L with an average value of 18.56 g/L. The statistical analysis of all verified values with p = 0.05 are stated in Table 2. The normal plot of the residuals of this model is displayed in Fig. 2 to evaluate the distribution of residuals. The simultaneous effect of algal biomass and fermentation time on bioethanol production at the central point of yeast volume % v/v is represented in Fig. 3, while the simultaneous effect of yeast volume % v/v and fermentation time at the central point of algal biomass on bioethanol production is exhibited in Fig. 4 and the simultaneous effect of algal biomass and yeast volume % v/v at the central point of time on bioethanol production is presented in Fig. 5. The predicted optimum value of bioethanol production after 43.6 h of fermentation time is 18.57 g/L; this value is predicted by fermenting 65 g/L algal biomass and 32.5 yeast volume % v/v.

Discussion

The merit of using the response surface method is to study more than one parameter simultaneously. However, selecting the parameters and their range values depends on their high significant effect on bioethanol production and their influence on each other. There is no doubts that high algal biomass need a high amount of yeast volume % v/v through adequate time to maximize the produced bioethanol. On the other hand, high bioethanol concentration causes yeast deterioration. This may happen via a long time process or high algal biomass concentration (Markou et al. 2013, Ho et al. 2013, Ashokkumar et al. 2015).

The fermentation yield % measured by Markou et al. (2013) is 56% by fermenting 12–13 g/L acidic hydrolysate biomass of *Antrosphira platensis*, while the fermentation yield % measured by Ho et al. (2013) is 90% by fermenting 10–80 g/L acidic hydrolysate biomass of *Chlorella vulgaris*. In this work, the fermentation yield % is 90% for fermenting 30 g/L of acidic hydrolyzed biomass in conditions of run 20. At the same conditions of biomass and yeast volume % v/v in run 15, the bioethanol production increases by increasing the fermentation time. Decreasing the yeast volume % in run 3 causes increasing bioethanol production. In this condition of the lowest biomass concentration, low yeast volume % and high time range increase the bioethanol production.

Fermenting the central value of 65 g/L algal biomass, the fermentation yield % is 90% in run 4 for fermentation time 88.36 h. The yield % increased to 94% as the time decreased to 48 h in run 7 at the same % yeast volume 32.5%. The yield % decreased to 61.93% as a consequence of increasing the yeast volume % to 61.93% in run 17. This reverse relation is implemented in run 1 too, where the yield % increases as the yeast volume % decreases.

The high border of algal biomass 100 g/L is fermented in various conditions. The bioethanol production records the minimum value of 11.46 g/L at high fermentation time

Table 3 Statistical analysis of the predicted solutions

	Prediction	95% CI low	95% CI high	95% PI low	95% PI high
Bioethanol production, g/L	18.57	18.32	18.83	18.08	19.07

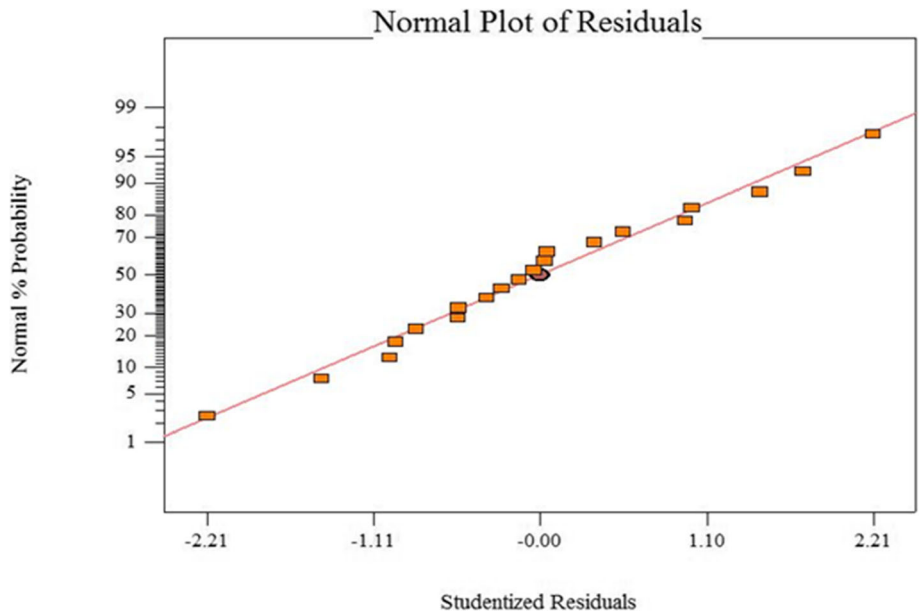

Fig 2 The normal plot of the residuals

of 72 h in run 19. The yield increase in run 2 is a consequence of decreasing the time to 24 h. Decreasing the yeast volume % to the minimum limit causes the increasing of the bioethanol production in run 14 at time 72 h and increased more in run 12 at time 24 h. According to the wide variance in the fermentation yield %, it is necessary to optimize the studied parameters simultaneously. The most effective route to optimize these parameters is RSM.

In this work, the interactive effect of algal biomass (*G*), fermentation time (*T*), and % yeast volume on the bioethanol production was investigated using RSM. The statistical analysis of the response revealed that the predicted R^2 is in harmony with the adjusted R^2. The analysis of variance (Table 2) for the partial sum of squares illustrated that the model *F*-value of 831.63 implies the model is significant and the values of Prob > *F* are less than 0.05 indicating that the model terms are significant, whereas the lack of fit *F*-value of 1.93 implies the lack of fit is not significant which indicates that the model can accurately predict the relationships between the reaction factors within the selected range.

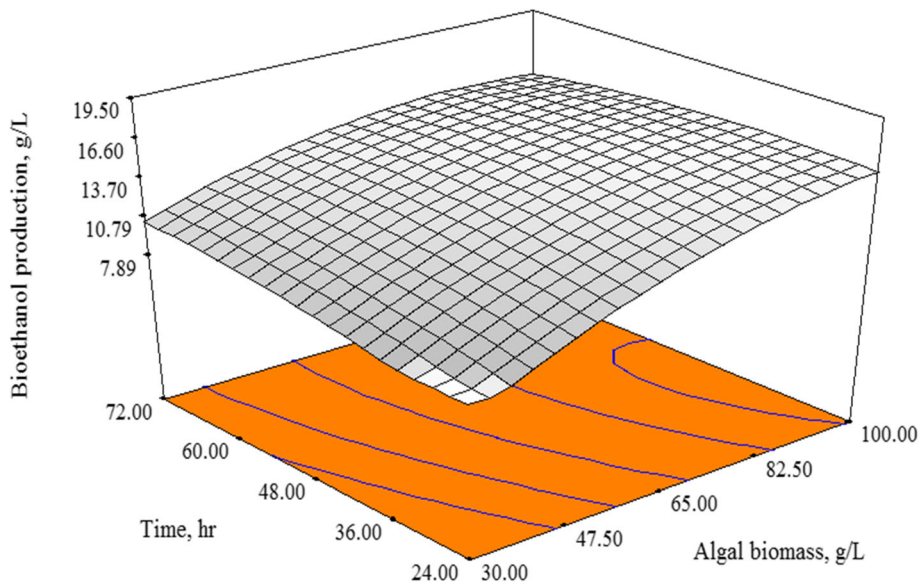

Fig. 3 The simultaneous effect of algal biomass and time on bioethanol yield at the central point of yeast volume % v/v

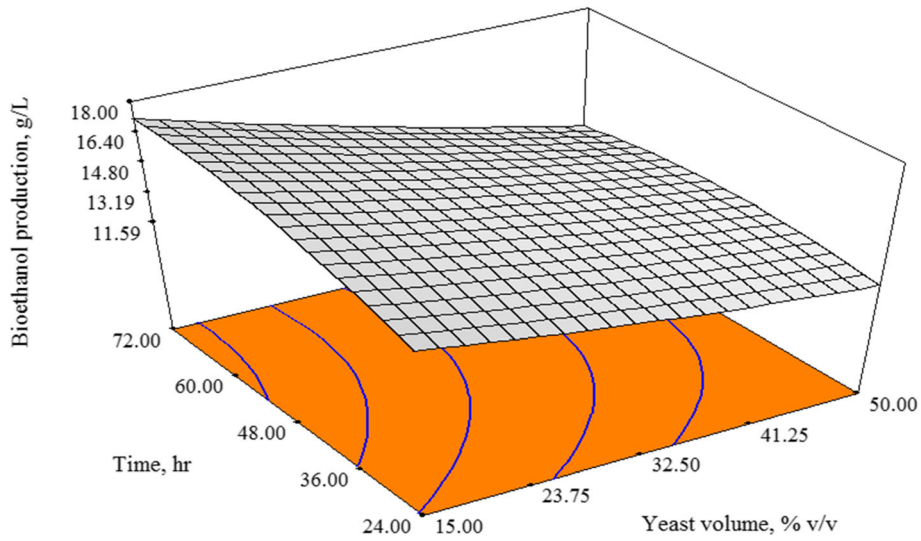

Fig 4 The simultaneous effect of yeast volume % v/v and time on bioethanol yield at the central point of the algal biomass

High variance is noticed as the coefficient of variation (C.V.) which is greater than 1 indicating that the data points are very spread out from the mean and from one another. The normal plots of the residuals are approximately linear verifying a normal distribution of the error terms. The optimum production of bioethanol was predicted with a prediction interval of 95% and a confidence interval of 95%.

The contour graphs in Figs. 3, 4, and 5 implemented the optimum values of this model. The maximum bioethanol production in Fig. 3 is 16.5–18.5 g/L at the central yeast volume % of 32.5 % v/v; this was achieved at a range of algal biomass of 90–100 g/L and fermentation time of 24–50 h. This is in agreement with Silva et al. (2018) since increasing the time factor increases the fermentation yield up to a limit of high bioethanol concentration relative to the yeast amount; this explains the declination of the curve beyond these values. The relation between the fermentation time and yeast volume % was exhibited in Fig. 4 where the trapped area of 40–72 h and 15–20 % v/v produced the highest bioethanol production (15–17 g/L) at the central algal biomass 65 g/L. This result is in harmony with El-Dalatony et al. (2016) and Silva et al. (2018), where the low yeast

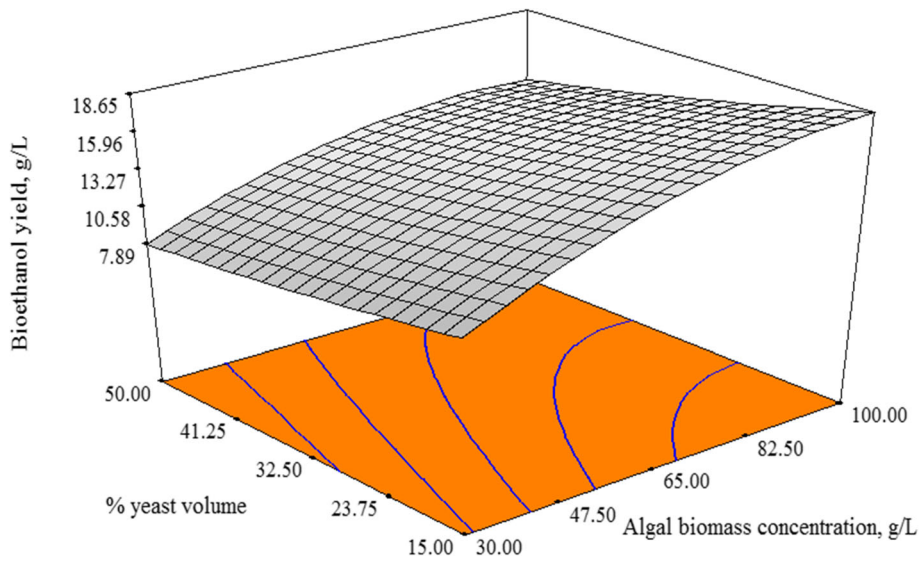

Fig 5 The simultaneous effect of yeast volume % v/v and algal biomass concentration on bioethanol yield at the central point of the time

volume % v/v requires higher time for bioethanol production. Finally, Fig. 5 shows the optimum conditions are 70–100 g/L and 15–20 yeast volume % v/v for bioethanol production (16–18.65 g/L) at the central point of time of 48 h. The combination of the three modules represented by the previous figures was solved by the Design-Expert program that implements the optimum area of the three parameters located in the range of 90–100 g/L, 15–20, and 40–50 h.

The optimum predicted bioethanol concentration calculated using RSM via the design expert program is 18.57 g/L through fermenting 98.7 g/L algal biomass by 15.09 yeast volume % v/v for 43.6 h fermentation time. The bioethanol to algae ratio is 0.188 g/g, while the ratio obtained by fermenting *Scenedesmus abundans* using 3% v/v *S. cerevisiae* for 48 h is 0.103 g/g (Guo et al. 2013), whereas fermenting *Chlorella vulgaris* using *Zymomonas mobilis* produces 0.178 g ethanol/g algae using SHF process (Ho et al. 2013). These lower values may be due to the small amount of yeast. On the other hand, fermenting *Chlorococum* sp. via *Saccharomyces bayanus* produces 0.38 g ethanol/g algae (Harun et al. 2010). This higher value was achieved at a small algal amount (10 g/L) and a high yeast amount (3 g/L) which is not applicable on a large scale. According to the lack of data about bioethanol productivity from *Microcystis* sp., it was effective to use RSM to minimize the number of experiments and perform an operative prediction model that describes the relationship between the studied parameters.

Conclusions

The effects of algal biomass, the fermentation time, and the yeast volume percent are three independent variables that greatly affect bioethanol productivity via anaerobic fermentation. This work optimizes these three individual parameters simultaneously in the range of 30–100 g/L algal biomass, 24–72 h fermentation time, and 15–50 yeast volume % v/v. The results revealed that the optimum conditions for fermentation are 98.7 g/L algae containing 45% carbohydrates, 15.09% immobilized yeast volume, and 43.6 h fermentation time in order to achieve 18.57 g/L bioethanol in a batch process. The prospective research is going to study the effect of these predicted parameters on continuous fermentation on the semi-pilot scale.

Abbreviations

C.V: Coefficient of variation; *G*: Algal biomass; HRAP: High rate algal pond; Prob > *F*: Probability function; R^2: Regression coefficient; RSM: Response surface methodology; SHF: Separate hydrolysis fermentation process; *T*: Fermentation time; *V*: yeast volume % v/v; WTP: Wastewater treatment plant

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Authors' contributions

SA studied the synergy of the results, performed statistical analyses, and applied the optimization using response surface methodology (RSM). SM treated the harvested algal biomass, hydrolyzed it by acid, followed up the fermentation process and performed the accompanying analyses. FA tackled the issues of *Saccharomyces cerevisiae* cultivation, storage, counting, propagation, immobilization and following up its count day by day during fermentation processes. GH is the PI of the project sponsored this work; she contributed to the design of the cultivation and the harvesting system of algae and supervised the whole work. All authors wrote and participated in the development and implementation of the research plan. All authors read and approved the final manuscript.

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Ethics approval and consent to participate

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Consent for publication

Not applicable

Competing interests

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