Electronic Journal of Biotechnology 38 (2019) 1–9

Contents lists available at ScienceDirect

Electronic Journal of Biotechnology

Research Article

Process conditions for a rapid *in situ* transesterification for biodiesel production from oleaginous yeasts

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ARTICLE INFO

Article history:
Received 26 February 2018
Accepted 28 November 2018
Available online 7 December 2018

Keywords:
Biodiesel
Biofuel
Environment
in situ transesterification
Methyl esters
Oleaginous microorganisms
Oleaginous yeast
Renewable energy
Response surface methodology
Rhodotorula graminis
Sustainable energy

ABSTRACT

Background: Microbial oils produced by diverse microorganisms are being considered as alternative sources of triglycerides for biodiesel production. However, the standalone production of biodiesel from microorganisms is not currently economically feasible. In case of yeasts, the use of low-value nutrient sources in microbial production and the implementation of cost-efficient downstream processes could reduce costs and make microbial lipids competitive with other commodity-type oils in biodiesel production. Industrial biodiesel synthesis from oleaginous seeds is currently based on a multistep process. However, a simple process called *in situ* transesterification (ISTE), which takes place within the biomass without a previous lipid extraction step, is receiving increasing interest. In this work, the optimal conditions for an ISTE process to obtain biodiesel from previously selected oleaginous yeast (*Rhodotorula graminis* S1/S2) were defined using the response surface methodology (RSM).
Results: Using the RSM approach, the optimal conditions for the maximum yield with minimum reaction time included a methanol-to-biomass ratio of 60:1, 0.4 M H_2SO_4, and incubation at 70°C for 3 h. The optimized *in situ* process yield was significantly higher (123%) than that obtained with a two-step method in which fatty acids from saponifiable lipids were first extracted and then esterified with methanol. The composition of the fatty acid methyl ester mixture obtained from *R. graminis* S1/S2 by ISTE met Uruguayan standards for biodiesel.
Conclusion: The characteristics achieved by the optimized method make microbial oil a potential alternative for biodiesel production from yeast at an industrial scale.
How to cite: Martinez-Silveira A, Villarreal R, Garmendia G, et al. Process conditions for a rapid *in situ* transesterification for biodiesel production from oleaginous yeasts. Electron J Biotechnol 2018;37. https://doi.org/10.1016/j.ejbt.2018.11.006.

1. Introduction

Biodiesel is a renewable and sustainable energy resource with several benefits compared with petro-diesel. It has a more favorable combustion emission profile, with no net emission of sulfur oxides, and low amounts of carbon monoxide, particulate matter, and unburned hydrocarbons [1]. Moreover, it has been demonstrated that it degrades in the environment faster than diesel fuel or gasoline [2].

Biodiesel consists of the alkyl esters of fatty acids that are typically produced by transesterification of triglycerides from vegetable oils with an alcohol (*e.g.*, methanol) in the presence of either a base or an acid catalyst. Overall production costs remain high, which constitutes a major limitation in commercial production and results in a more expensive product than fossil fuels. The price of oil feedstock, which represents approximately 70–90% of the total cost [3], has been recognized as the main reason for high prices. Moreover, concern has been raised about the risk of diverting farmland or food crops to biofuel production to the detriment of the food supply. To overcome these problems, alternative sources of triglycerides, such as waste, nonedible, and microbial oils are being considered. In particular, microbial oils, produced by oleaginous microorganisms, are similar in composition as that of oils from oleaginous seeds and therefore suitable to be used for biodiesel production. Moreover, microbial oil production is not limited by land availability, is less affected by season and climate, and can be more easily scaled up than plant oil production [4]. Oleaginous microorganisms defined as organisms in which the lipid content exceeds 20% of their dry weight, include microalgae, yeast, fungi, and bacteria. In case of yeasts, there are many studies reporting that under certain culture conditions, lipid accumulation

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Peer review under responsibility of Pontificia Universidad Católica de Valparaíso.

https://doi.org/10.1016/j.ejbt.2018.11.006

Table 1
Variables and their levels used in the factorial design.

Factor	Level		
	low (−1)	medium (0)	high (1)
Catalyst concentration [H_2SO_4], M	0.20	0.30	0.40
Temperature (°C)	40	55	70
Time (hours)	5	12.5	20
Methanol–biomass ratio (*v*/*w*)	20	30	40

could range from 30 to 50% of their dry cellular biomass [5]. Intracellular yeast lipids mainly include triacylglycerides [6] and are considered promising candidates as sustainable biodiesel precursors. However, the standalone production of biodiesel from yeast is not currently economically feasible [7]. The use of low-value nutrient sources in yeast production and the implementation of cost-efficient downstream processes could reduce costs and make yeast lipids competitive with other commodity-type oils in biodiesel production [8].

Industrial biodiesel synthesis from oleaginous seeds currently involves isolation of glycerides by extrusion or solvent extraction, degumming, refining of the oil, and transesterification [9]. In case of microbial oils, a similar process involving lipid extraction and subsequent acid or base-catalyzed transesterification has been used to produce biodiesel from biomass. The selection of the catalyst in a transesterification process is dependent on the feedstock to be used [10]. Base-catalyzed processes require a feedstock with low free fatty acid (FFA) content, not exceeding 0.5% of the total lipid weight [10], and anhydrous glycerides and alcohol are necessary to avoid saponification. They are in general faster and usually occur at lower temperatures than acid-catalyzed processes, but they could not be used for feed stocks with high content of FFAs [11,12]. Currently, a simple process called *in situ* transesterification (ISTE), which occurs in a single step, directly from yeast biomass, without a previous lipid extraction, is being investigated [13]. Such a process has been developed by Liu and Zhao [14] and Thliveros et al. [15] to obtain biodiesel from *Rhodotorula toruloides* and *Lipomyces starkeyi*. The present work proposes the development of an ISTE process to obtain biodiesel from *Rhodotorula graminis* S1/S2, an oleaginous yeast previously selected for its high capacity to store intracellular saponifiable lipids [16]. For this purpose, the type of catalyst was selected and the optimal conditions for the ISTE were identified using the surface of the response methodology.

2. Materials and methods

2.1. Strain, culture, and harvesting conditions

R. graminis S1/S2, a type of oleaginous yeast selected by Pereyra et al. [16] for its capacity to store intracellular saponifiable lipids under certain culture conditions, was used throughout this study. The yeast was grown at 28°C in a rotary shaker at 150 rpm in 250 mL Erlenmeyer flasks containing 50 mL of liquid nitrogen-limited medium developed by Thakur et al. [17] for intracellular lipid accumulation. The medium consisted of the following (g L^{-1}): glucose, 40; KH_2PO_4, 0.75; yeast extract, 1.5; NH_4NO_3, 0.285; $CaCl_2.2H_20$, 0.4; and $MgSO_4.7H_20$, 0.4 [17]. The pH of the medium was initially adjusted to pH 5.0. Each flask was inoculated with 5 mL of yeast suspension (turbidity = McFarland 1) prepared in sterile distilled water from a 48 h culture at 28°C in potato dextrose agar (PDA). After 5 d of incubation, cultures were centrifuged and pellets were washed with distilled water.

Table 2
Variables and their levels used in the factorial design.

Factor	Level		
	low (−1)	medium (0)	high (1)
Catalyst concentration [H_2SO_4], M	0.40	0.50	0.60
Time (hours)	3	5	7
Methanol–biomass ratio (*v*/*w*)	40	50	60

2.2. Two-step procedure for the transesterification of yeast lipids

Methyl esters from intracellular yeast lipids (fatty acid methyl esters, FAMEs) were obtained by a two-step procedure, which implied the extraction of fatty acids from the saponifiable fraction of intracellular lipids followed by esterification with methanol. Cells from 40 mL of yeast culture obtained as described above were collected by centrifugation, washed with distilled water, and dried for 24 h at 70°C. Saponifiable lipids from the biomass were determined as described by Pereyra et al. [16]. The dried pellet of cells was treated with 5 mL of KOH 30% *w*/*v* and 5 mL of ethanol 95% *v*/*v* and incubated overnight at 70°C. Unsaponifiable matter of the resulting solution was removed with hexane. The pH of the remaining aqueous phase was adjusted to 1 with HCl, and then, fatty acids were extracted twice with 10 mL of hexane. After hexane evaporation under reduced pressure, the solid residue obtained, which represented the fatty acid fraction from saponifiable lipids, was weighed. At the same time, in each case, biomass of yeasts was determined as the dry weight of cells contained in 2 mL of culture. To obtain methyl esters, the saponifiable lipids were resuspended in 1 mL of hexane. An aliquot corresponding to 20 mg of fatty acids was transferred to a glass tube, and the solvent was evaporated. Then, the esterification of the resulting fatty acids with methanol was carried out as described by Burja et al. [18]. Briefly, 3 mL of methanol:HCl:chloroform (10:1:1) *v*/*v*/*v* was added to 20 mg of the fatty acid mixture and heated during 2 h at 90°C. Afterwards, 1 mL of distilled water was added to the mixture, and the methyl esters were extracted three times using 2 mL of hexane: chloroform (4:1) *v*/*v*. Water residue from the organic phase containing the methyl esters was removed by adding 0.5 g of anhydrous Na_2SO_4.

2.3. Direct methanolysis of yeast lipids

Direct methanolysis catalyzed by an acid [14] or a base [15] for the transesterification of yeast lipid into FAMEs was compared. Yeast biomass obtained as described above was washed and dried for 24 h at 70°C. In each case, 55 mg of the dried biomass was weighed and then dispersed in an acidic ([H_2SO_4] = 0.2 M) or a basic ([NaOH] = 5 g/L) methanolic solution, thus maintaining the biomass:methanol ratio (1:20, *w*/*v*). The mixture was incubated at 70°C for 20 h and at 50°C for 10 h for acid- or base-catalyzed transesterification, respectively. The obtained methyl esters were extracted from the mixture using hexane:chloroform (4:1, *v*/*v*) according to Burja et al. [18] as described above. The methyl esters obtained in both cases were compared with those obtained by the two-step procedure using one-way ANOVA, and mean separations were performed by HSD tests. Differences at $P < 0.05$ were considered significant.

2.4. Analysis of the fatty acid methyl esters

The analysis of FAMEs was conducted by gas chromatography with a flame ionization detector (GC-FID) using Agilent Technologies 7820A GC equipped with a DB-225 (20 m, 0.100 mm ID, 0.10 μm) column and a flame ionization detector. The operation condition was N_2 carrier gas 0.2 mL/min and the injection port and detector temperature was 250°C. The following temperature program was applied: 40°C for 0.5 min, increase of 25°C/min to 195°C, increase of 3°C/min to 205°C, increase of 8°C/min to 230°C, and hold at 230°C for 10 min. Methyl nonadecanoate (74208-1G methyl nonadecanoate analytical standard Fluka Analytical Sigma-Aldrich 1891) was used as an internal standard at a concentration of 1.5 mg/mL. FAMEs were identified by comparison of retention times with those obtained with known standards (Supelco

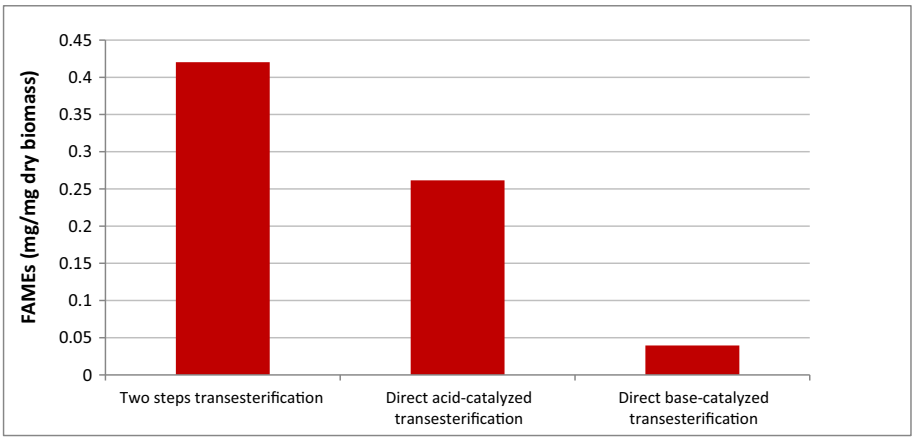

Fig. 1. FAME yields of the different transesterification reactions; the experimental data were analyzed for statistical significance using one-way ANOVA, with comparison using Tukey's HSD (honestly significant difference) test to determine significant differences. Differences were considered significant at the level of $P < 0.05$. Significant differences as revealed by Tukey's HSD test are indicated by different letters above the bars.

37 Component FAME Mix; Sigma-Aldrich 1891) separated under the same GC conditions. Total methyl esters were calculated as the sum of each individual peak area related to the area of the internal standard.

2.5. Optimization of direct acid-catalyzed transesterification

The optimization of the direct acid-catalyzed transesterification process from yeast biomass was carried out using two sequential full-factorial designs. The designs aimed to assess the effect of different factors on the amount of fatty acid methyl esters obtained from a determined amount of yeast biomass. The first step involved four factors (catalyst concentration, methanol–biomass ratio, temperature, and time of reaction). Each factor was examined at two levels (−1 for the low level and +1 for the high level) and at the central point (0). The total number of experiments was 19 ($2^4 + 3$), as three replicates of the central point were performed. The factor levels studied are listed in Table 1.

Next, a 2^3 full-factorial design was carried out to extend the range of values of three factors according to the results obtained in the previous experiment. The factors involved in the second factorial design were catalyst (H_2SO_4) concentration, methanol–biomass ratio, and reaction time. The levels of these factors used in the design are shown in Table 2.

In this case, 11 trials were performed ($2^3 + 3$), as three replicates of the central point were included. In both factorial designs, after reactions were completed, methyl esters were extracted and quantified by GC as described above. Experimental design and statistical analysis were performed using Design-Expert 7.0 software (Stat-Ease Inc.). Variables with *P*-values lower than 0.05 were considered to have a significant effect on the transesterification process. A reaction under the optimized conditions was performed to validate correctness of the computational model.

2.6. Optimization of reaction time

According to the results obtained from factorial designs, a complementary experiment was carried out to optimize the reaction time. Transesterification reactions from dried yeast biomass were performed (in duplicate) using H_2SO_4 0.4 M, a methanol–biomass ratio of 60, and heating at 70°C for 1, 2, 3, 5, or 7 h. The methyl esters obtained in both cases were compared using one-way ANOVA, and mean separations were performed using HSD tests. Differences at $P < 0.05$ were considered significant. The methyl esters obtained from the optimum reaction time were compared with those obtained by the two-step procedure.

Table 3
Factorial design experiment design matrixes and the response values.

Run	Catalyst concentration [H_2SO_4], M (A)	Temperature (°C) (B)	Time (hours) (C)	Methanol–biomass ratio (*v*/*w*) (D)	FAME (mg/mg of dry biomass)
1	0.4	70	5	40	0.512
2	0.2	40	5	40	0.350
3	0.2	70	20	20	0.241
4	0.4	40	20	40	0.261
5	0.2	70	5	20	0.301
6	0.2	40	20	40	0.255
7	0.2	70	5	40	0.372
8	0.3	55	12.5	30	0.250
9	0.3	55	12.5	30	0.276
10	0.2	40	20	20	0.176
11	0.2	40	5	20	0.137
12	0.4	40	5	40	0.153
13	0.4	70	20	20	0.577
14	0.4	40	5	20	0.165
15	0.3	55	12.5	30	0.230
16	0.4	40	20	20	0.158
17	0.4	70	5	20	0.338
18	0.2	70	20	40	0.282
19	0.4	70	20	40	0.569

Table 4
ANOVA showing the effect of factors and their interactions for FAMEs concentration in the factorial design.

Source	Sum of squares	df	Mean square	F value	*P*-value Prob > F	
Model	0.28	6	0.047	17.76	<0.0001	significant
Catalyst concentration [H_2SO_4] (A)	0.024	1	0.024	8.98	0.0121	
Temperature (B)	0.15	1	0.15	55.38	<0.0001	
Time (C)	0.000228	1	0.000228	0.86	0.3749	
Methanol–biomass ratio (D)	0.027	1	0.027	10.24	0.0084	
AB	0.06	1	0.06	22.56	0.0006	
AC	0.023	1	0.023	8.52	0.0139	
Curvature	0.000656	1	0.000656	2.46	0.1452	
Residual	0.029	11	0.32			
Lack of Fit	0.028	9	0.000314	5.9	0.1536	not significant
Pure Error	0.000106	2	0.0000532			
Cor. Total	0.32	18				

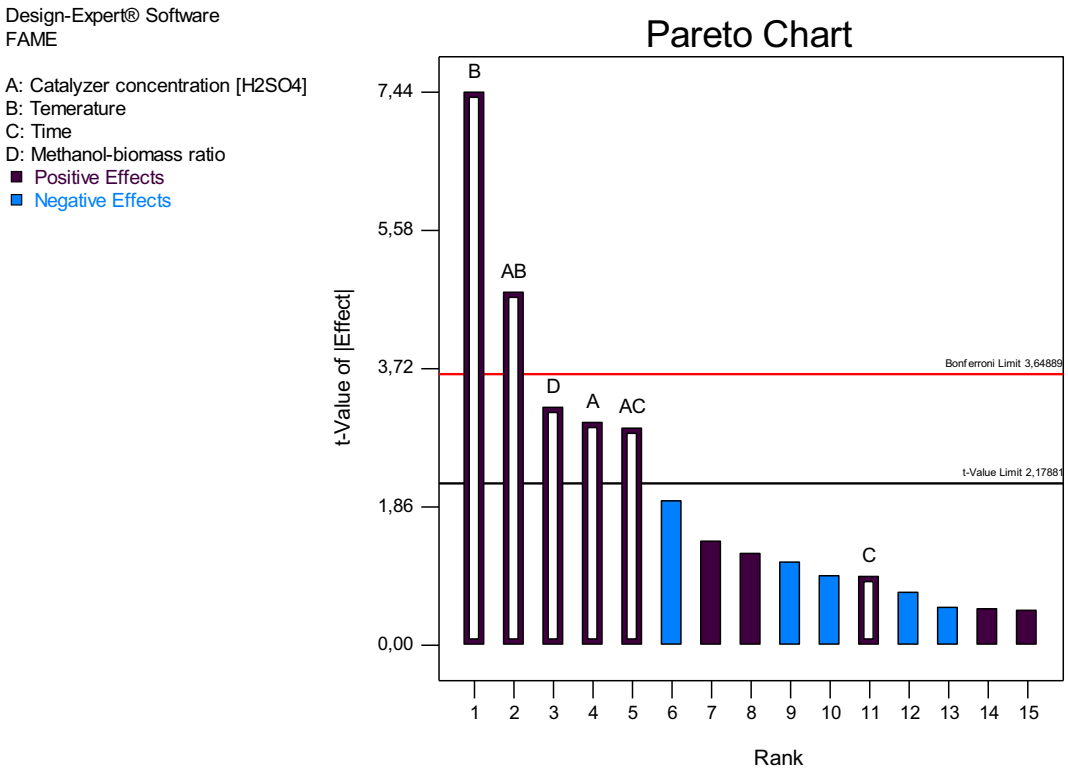

Fig. 2. Pareto charts obtained after the factorial design showing contribution of factors *vs.* their main effect on the concentration of FAME.

2.7. Evaluation of the optimized method in a different oleaginous yeast species

Candida glaebosa 12D, a type of oleaginous yeast isolated from the sub-Antarctic region [19], was grown at 20°C under the same conditions described above to obtain intracellular lipid accumulation by *R. graminis* S1/S2. After 5 d of incubation, cultures were centrifuged and pellets were washed with distilled water and dried at 70°C for 24 h. Fifty-five milligrams of the dried biomass was weighed, and FAMEs were determined directly from biomass by the ISTE process optimized in this work or from saponifiable lipids extracted from biomass by the two-step procedure described above. Each treatment

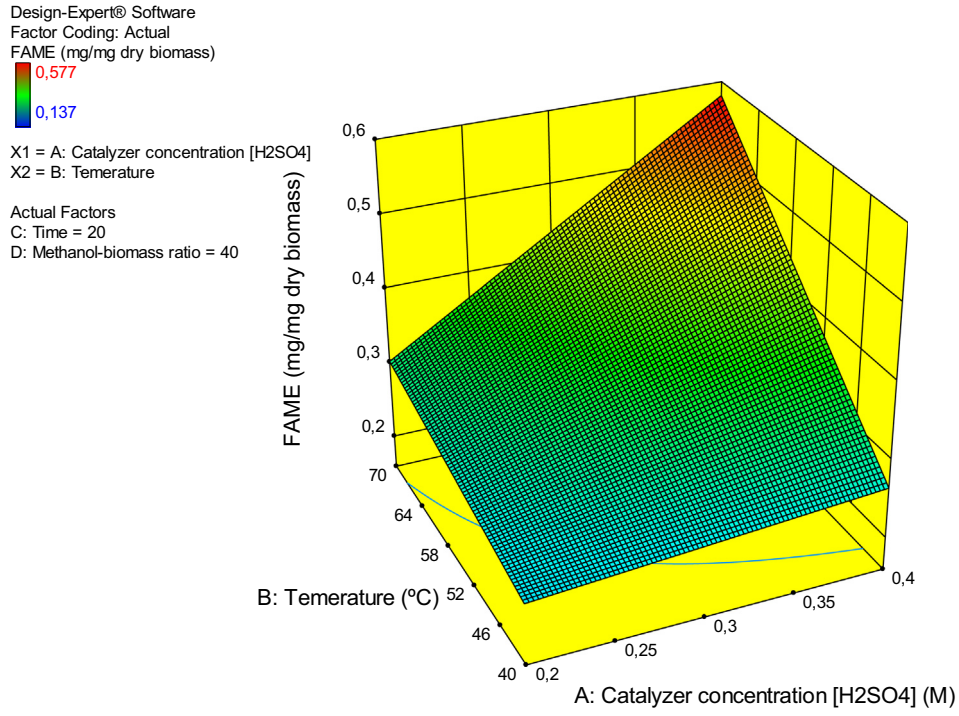

Fig. 3. Response surface showing the relation between the concentration of FAMEs, temperature, and catalyst concentration in the factorial design range.

Table 5
Factorial design experiment design matrixes and the response values.

Run	Time (hours) (A)	Catalyst concentration [H_2SO_4] M (B)	Methanol–biomass ratio (*v*/*w*) (C)	FAME (mg/mg of dry biomass)
1	5	0.5	50	0.454
2	7	0.4	60	0.516
3	3	0.6	40	0.341
4	7	0.4	40	0.545
5	5	0.5	50	0.436
6	5	0.5	50	0.429
7	7	0.6	60	0.447
8	3	0.4	40	0.378
9	3	0.6	60	0.420
10	7	0.6	40	0.467
11	3	0.4	60	0.544

was performed in duplicate. The methyl esters obtained in both cases were compared using one-way ANOVA, and mean separations were performed by HSD tests. Differences at $P < 0.05$ were considered significant.

3. Results

3.1. Microbial biomass production

Intracellular saponifiable lipids were accumulated by *R. graminis* S1/S2 under the culture conditions used in this work. Fatty acids corresponding to those lipids represented 0.440 ± 0.010 mg per mg of dry biomass, which corresponds to 44% *w*/*w* of cell dry weight. All subsequent experiments were carried out using the same yeast biomass.

3.2. Comparison of the two-step transesterification process with acid- and base-catalyzed methanolysis from yeast biomass

FAMEs corresponding to fatty acids of intracellular saponifiable lipids obtained using the two-step process were 0.400 ± 0.032 mg of FAMEs per mg of dry biomass. In case of base- and acid-catalyzed ISTE, the amount of FAMEs per mg of dry yeast biomass was 0.0396 ± 0.0002 and 0.261 ± 0.012, respectively (Fig. 1). The results were compared by one-way analysis of variance (ANOVA), and mean separations were performed by HSD tests using Infostat software [20]. Differences at $P < 0.05$ were considered significant. As shown in Fig. 1, the amount of FAMEs obtained by acid-catalyzed ISTE was significantly higher than that obtained by the base-catalyzed method; however, the yield was significantly lower than that obtained by the two-step transesterification process.

Table 6
ANOVA showing the effect of factors and their interactions for FAME concentration in the factorial design.

Source	Sum of squares	df	Mean square	F value	*P*-value Prob > F	
Model	0.038	4	0.009533	20.56	0.0026	significant
Time (A)	0.011	1	0.011	23.1	0.0049	
Catalyst concentration [H_2SO_4] (B)	0.012	1	0.012	25.51	0.0039	
Methanol–biomass ratio (C)	0.000484	1	0.000484	10.44	0.0232	
AC	0.011	1	0.011	23.17	0.0048	
Curvature	0.0000686	1	0.0000686	1.48	0.2782	
Residual	0.000232	5	0.0000464			
Lack of Fit	0.000199	3	0.0000662	3.97	0.2075	Not significant
Pure Error	0.0000333	2	0.0000167			
Cor. Total	0.041	10				

3.3. Optimization of direct acid-catalyzed transesterification

Table 3 shows the amount of FAMEs obtained in the 2^4 factorial design.

Data were evaluated using ANOVA, and a model involving significant factors was generated. The results are shown in Table 4.

The model showed significance ($F = 17.76$; $P < 0{,}0001$) with an R2 value of 0.8876, meaning that it could explain 88.76% of the variability in the response. The predicted R2 was in reasonable agreement with the adjusted R2 value of 0.8313, thus indicating an acceptable agreement between the experimental and predicted values for the concentration of FAMEs. The adjusted R2 corrected the R2 value for the sample size and the number of terms in the model. If there are many terms in the model and the sample size is too small, the adjusted R2 may be noticeably smaller than R2 [21]. Lack of fit and curvature were not significant in the model. Three factors (temperature, catalyst (H_2SO_4) concentration, and methanol–biomass ratio) had a significant influence ($P > 0.05$) on FAMEs obtained from the yeast biomass. The interactions between the catalyst concentration and temperature or time also showed significance. Methanol–biomass ratio and interactions between catalyst concentration and temperature or time had a positive effect on the response, while catalyst concentration, temperature, and time showed a negative effect. However, reaction time was not statistically significant; therefore, it was fixed at the minimum value of the studied range (5 h) in the next stage of optimization (Table 2). Fig. 2 shows the Pareto chart representing the main and interaction effects of factors involved in the process. It clearly shows that the *t*-value of effects corresponding to temperature and the interaction between temperature and the catalyst (H_2SO_4) concentration exceeds the Bonferroni limit of 2.776, and hence, they have certainly significant effects for obtaining FAMEs. Moreover, the effects corresponding to the catalyst concentration, the methanol–biomass ratio, and the interaction between the catalyst concentration and time were likely to be significant, as *t* values of their effects were between the Bonferroni and *t*-value limit [22]. All the mentioned factors were included in the model. The response surface plot generated (Fig. 3) represents the simultaneous effect of two variables (temperature and catalyst concentration) on the concentration of FAMEs, taking the other two variables at a constant level. On observing the plots, it can be concluded that the highest levels of FAMEs were achieved when the highest catalyst concentration and temperature was used. Moreover, higher amounts of FAMEs were obtained with a higher methanol–biomass ratio. As time did not have a significant effect on the response, we decided to minimize reaction time to have a shorter and less expensive process. With these criteria, the optimized conditions to maximize the FAME concentration were as follows: temperature, 70°C; catalyst concentration, 0.4 M; methanol–biomass ratio, 40; and reaction time, 5 h. According to the model, a predicted FAME concentration of 0.483 mg/mg of dry biomass would be achieved. The result of this condition corresponded to one of the replicates of the factorial; hence, it could be compared with the obtained experimental value (0.512 mg of FAMEs per mg of dry biomass). That result validated the model, as a deviation of only 6% from the theoretical value was obtained. To determine the effect of higher values of two significant variables (catalyst concentration and methanol–biomass ratio) in the obtainment of FAMEs, another 2^3 full-factorial design was carried out. The reaction time was included in the model to determine whether shorter times would have an effect on the response. Variable temperature was not included in the design to avoid problems caused by excessive methanol evaporation. Table 5 shows the amount of FAMEs obtained in the 2^3 factorial design experiments, which were in the range of 0.341 to 0.544 mg of FAMEs per mg of dry biomass.

ANOVA results, as shown in Table 6, was used to determine the significance of the model ($F = 20.56$, $P = 0.0026$).

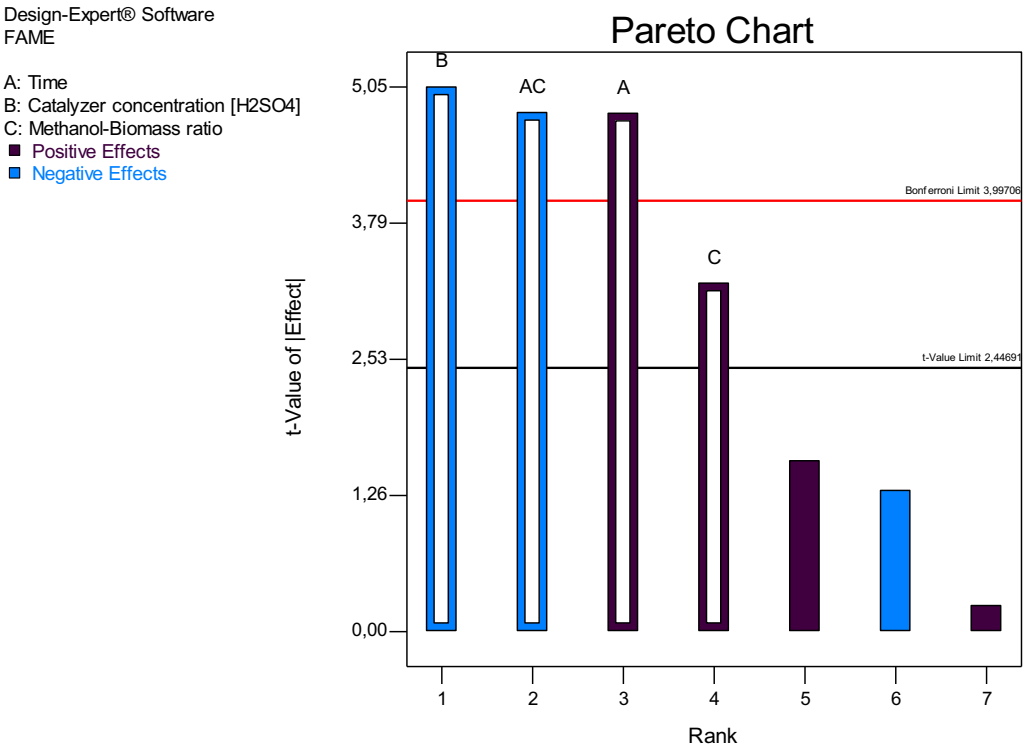

Fig. 4. Pareto charts obtained after the factorial design showing contribution of factors *vs.* their main effect on the concentration of FAME.

The lack of fit and curvature were not significant, with *P*-values of 0.2075 and 0.2782, respectively. R2 obtained from this analysis was 0.9270, which indicates that the model fits the experimental and predicted values well. The Pareto chart obtained in this case is shown in Fig. 4. The *t*-value of the effect corresponding to the methanol–biomass ratio, catalyst concentration, time, and the interaction between time and methanol–biomass ratio was above the *t*-value limit line; hence, they were considered as significant factors in the concentration of FAMEs. Reaction time and methanol–biomass ratio had a positive effect on the response, while the catalyst concentration

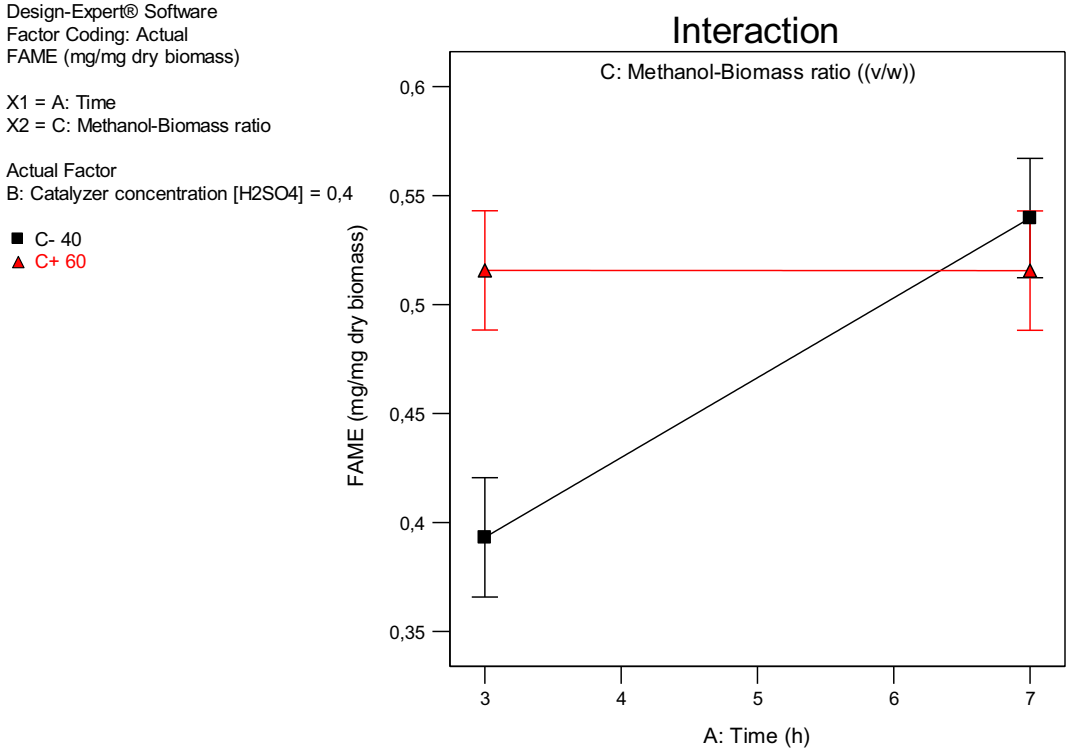

Fig. 5. Interaction between methanol-to-biomass ratio and time. Catalyst concentration, 0.4 M.

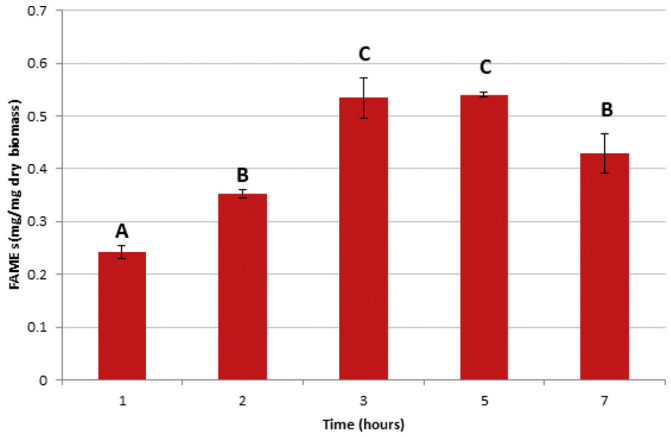

Fig. 6. FAMEs of the different reaction time; experimental data were analyzed for statistical significance using one-way ANOVA, with comparison using Tukey's HSD (honestly significant difference) test to determine the significant differences. Differences were considered significant at the level of $P < 0.05$. Significant differences as revealed by Tukey's HSD test are indicated by different letters above the bars.

and the interaction between time and methanol–biomass ratio showed a negative effect, which was indicated by a positive or negative coefficient for each factor in the following first-order model, which could explain FAME concentration:

$$\begin{aligned}\text{FAME concentration} = {} & -0.027794 + (0.10993^{*}\ \text{Time}) \\ & -(0.38456^{*}\text{catalyzer concentration}) \\ & +(0.011623^{*}\ \text{methanol–biomass ratio}) \\ & -(-1.83256\text{E}{-}003^{*}\ \text{Time}^{*}\ \text{methanol–biomass ratio})\end{aligned}$$

According to the model, to get a higher amount of FAMEs from dry yeast cell, the process involving heating at 70°C should be carried out using the lowest catalyst concentration (0.4 M), the highest methanol–biomass ratio (60), and the longest reaction time (7 h) within the value ranges used for each variable in the design. However, when the interaction between the last two factors was analyzed (Fig. 5), it seemed that time was significant only when a low methanol–biomass ratio was used. When a ratio of 60 was used, reaction time was not significant.

3.4. Optimization of reaction time

Fig. 6 shows the FAMEs obtained when transesterification was carried at conditions of 70°C, 0.4 M catalyst concentration, and methanol–biomass ratio 60 at different reaction times (1, 2, 3, 5, and 7 h). The results were compared using one-way ANOVA, and mean separations were performed using HSD tests. Differences at $P < 0.05$ were considered significant. As shown in Fig. 6, the highest yields (0.534 ± 0.055 and 0.540 ± 0.006 mg FAMEs per mg of dry biomass) were obtained with reaction times of 3 and 5 h, respectively. The yields did not show significant difference between them but showed significant difference for those obtained with lower and higher reaction times. To minimize costs and time, a process of 3 h was selected. Under optimized conditions, FAME yield increased almost to double with regard to the initial conditions. Moreover, an important reduction in the reaction time was achieved. When the yield was compared to that obtained with the two-step process, a significant increase (21%) was also achieved. Table 7 shows the FAME profiles of the two-step transesterification and the direct acid-catalyzed transesterification with the cetane number used in both reactions.

Table 7
FAME profiles obtained by two-step transesterification and direct acid-catalyzed transesterification.

	Two-step transesterification	Direct acid-catalyzed transesterification
%14:0	1.07 ± 0.28	0.73 ± 0.06
%16:0	16.8 ± 2.9	14.60 ± 0.39
%16:1	3.10 ± 0.97	1.11 ± 0.04
%18:0	1.44 ± 0.10	2.77 ± 0.06
%18:1	60.7 ± 1.7	73.95 ± 0.53
%18:2	6.37 ± 0.26	1.64 ± 0.28
%18:3	1.62 ± 0.08	0.54 ± 0.04
%20:0	2.56 ± 0.95	0.56 ± 0.021
%24:0	0.30 ± 0.06	0.73 ± 0.07
Cetane number	58	58

3.5. Evaluation of the optimized method in a different oleaginous yeast species

FAMEs obtained from the dry biomass of *C. glaebosa* 12D with the two-step transesterification described above represented 0.56 ± 0.07 mg per mg of dry biomass, which corresponds to 56% *w/w* of cell dry weight. When FAMEs were obtained from the same biomass using the ISTE process optimized in this work, 0.61 ± 0.04 mg of FAMEs per mg of dry biomass were recovered, which represented the 108% of the amount obtained with the two-step procedure. However, when yields obtained by both methods were compared using one-way ANOVA, they did not show significant difference between them.

4. Discussion

In this work, optimal conditions to obtain biodiesel from *R. graminis* S1/S2 by an ISTE process were established. By heating dry yeast biomass for 3 h at 70°C in the presence of methanol (methanol–biomass ratio = 60) in the presence of 0.4 M H_2SO_4 as the catalyst, 0.534 mg of FAMEs per mg of dry yeast biomass was obtained. This yield was significantly higher (123%) than that obtained with the two-step process (lipid extraction followed by transesterification) used in this work. As ISTE is a one-step process that minimizes possible losses due to transfers, higher yields can be expected with regard to the two-step process. In addition, obtaining larger amounts of FAMEs could also be due to better lipid extraction achieved in the optimized ISTE process. Our results are in agreement with those of Harrington and D'Arcy-Evans [13] who reported that higher FAME yields from sunflower seeds were obtained when an ISTE process was used in comparison with conventional methods involving more steps. Different ISTE methods with both base and acid catalysts have also been employed to obtain biodiesel from microorganisms, including algae, fungi, and yeasts [3,14,23,24,25]. In the case of yeasts, Liu and Zhao [14] developed an acid-catalyzed ISTE process involving biomass heating at 70°C for 20 h, obtaining a FAME yield of 96.8% with regard to neutral lipids in the cells of the yeast *Lipomyces starkeyi*. They also tested a base-catalyzed process resulting in yields lower than 20% for the same yeast. However, Thliveros et al. [15] reported higher FAME yields (97.7%) from the dry biomass of *R. toruloides* when using a base-catalyzed process for 10 h at 50°C. Both ISTE processes, acid- and base-catalyzed, were assayed in this work to obtain biodiesel from the dry biomass of *R. graminis* S1/S2. In our case, the acid-catalyzed process developed by Liu and Zhao [14] rendered higher FAME yields (60%) than the base-catalyzed method (9%) with regard to FAMEs obtained from total saponifiable lipids. The efficiency of a base-catalyzed method greatly depends on the content of FFA in the feedstock as pointed out by Go et al. [26]. In fact, an alkaline catalyst is normally not recommended if the feedstock contains more than 2% of FFAs per total lipids due to saponification reactions that could interfere with the transesterification process [27]. Significant amounts (approximately 5% of total lipid) of FFA have been commonly found in yeast biomass [29]. However, a higher FFA content maybe found if yeasts are harvested after the carbon source in the growing medium has been depleted or during the storage time of the wet biomass by

hydrolysis of triglycerides in the intracellular lipid bodies [26]. In that sense, Suzuki and Hasegawa [30] reported that when the oleaginous yeast *Lipomyces starkeyi* reached the stationary phase and the medium became glucose deficient, it began to consume its own fat causing a decrease in triglycerides and an increase in intracellular FFAs, which reached 21% of the total lipid content. The use of a base-catalyzed ISTE is usually preferred due to the lower reaction times and temperature [11,12]; however, the type of feedstock represents an important factor to be considered in selecting the method for biodiesel production.

In this work, an acid-catalyzed ISTE process was selected to obtain biodiesel from *R. graminis* S1/S2, after 5 d of growth in the medium defined by Thakur et al. [17]. On the basis of the ISTE process designed by Liu and Zhao [14], two experimental designs were conducted to understand the impact of process variables in the obtainment of FAMEs. A significant improvement of more than 21% in FAME yield was obtained with regard to the initial process. Four process variables (catalyst concentration, methanol–biomass ratio, temperature, and time of reaction) were studied. According to Chopra et al. [28], an increase in the reaction temperature reduces the viscosity of lipids and enhances reaction rate but only to a critical level, beyond which the FAME yield is reduced. Generally, acid-catalyzed ISTE reactions are carried out at relatively high temperatures, near or just above the boiling point of the alkyl donor [26,31], which in the present work was represented by methanol (boiling point is 64.7°C). On the basis of previous works that used 70°C as the reaction temperature [14], in the present work, temperatures between 40°C and 70°C were assayed to obtain the optimum conditions for the ISTE process. The temperature selected was the maximum assayed (70°C), which coincides with the temperature used by Liu and Zhao [14]. The best methanol–biomass ratio (*v*/*w*) was set at 60, which represents a methanol–triglyceride molar ratio of 2467, calculated considering an average molar weight of 296 (MW of methyl oleate) for the triglycerides in biomass. This ratio resulted higher than that used in previous works with yeasts [14,15] but lower than the ratios used in ISTE processes with microalgae, reaching values near to 3400 [31]. As methanol plays a dual role in ISTE, acting as an extractant and as a reagent (alkyl donor), such processes normally require methanol–triglyceride molar ratios of several hundreds or thousands as opposed to the conventional transesterification of refined oil where a twofold molar excess of alcohol over total fatty acid content is generally sufficient [32,33]. In both cases, methanol is used in excess; hence, after the reaction is complete, the residual methanol must be recovered to be reused in the next batch, which is essential to minimize operating costs [34]. Sulfuric acid was the catalyst selected for ISTE reactions at a concentration of 0.4 M. Some previous ISTE methods have used sulfuric acid as the catalyst in concentrations between 2% and 4% (*v*/*v*) [14,28]. In our case, the selected concentration is equivalent to 3.92%, in accordance with that selected by Chopra et al. [28] to obtain biodiesel from the yeast *Pichia guilliermondii* by an ISTE process. In the present work, a significant reduction in reaction time was achieved with regard to previously developed ISTE processes such as those reported by Liu and Zhao [14], and by Chopra et al. [28]. In fact, the reaction time in our process was shorter by 6 and 2 times, respectively. Moreover, this process also involves shorter reaction times than those used by other ISTE methods involving alkaline catalysis [15] and by two-step methods [18]. The development of a shorter process with similar or higher FAME yields is an important achievement of the present work, as it will surely have a significant effect on the overall costs of biodiesel production from yeast biomass. The main cost of the ISTE method developed here is associated with the drying process before transesterification. However, according to Chopra et al. [28], the energy consumption associated with biomass drying in an oven would be less than that required for cell disruption if wet biomass was used as the feedstock.

The FAME profile found that the ISTE and the two-step transesterification processes used in the present work was very similar, with oleic and palmitic esters being the main components. The percentage of oleic ester was significantly higher after the ISTE process, with a concomitant reduction in the proportion of the other esters except for lignoceric and stearic acids, which were recovered in a greater proportion. Biodiesel must meet certain requirements to be used as a fuel, some of which rely on the fatty acid mixture used in biodiesel production. In Uruguay, standards for biodiesel indicate that the cetane number must be higher than 45, and no more than 12% of linolenic acid esters or 1% of esters from polyunsaturated acids (more than 4 double bonds) is admitted [35]. The fatty acid profiles from *R. graminis* S1/S2 obtained by the optimized ISTE complied with the Uruguayan standards for biodiesel. The ISTE method optimized in this work was also useful for obtaining FAMEs from intracellular lipids of *C. glaebosa* 12D, an ascomycetous yeast strain previously selected by our group. As *R. graminis* is a basidiomycetous yeast, the method could be considered applicable to yeasts of both Ascomycota and Basidiomycota phyla. However, the yields obtained with other oleaginous species of both phyla should be determined to evaluate the scope of the method.

ISTE processes, such as the one described in this work, are also being studied to obtain biodiesel from oil seeds, as they involve fewer steps than the conventional processing. Solvent extraction of oils, which is required in the conventional process and is cost-intensive [36], is not necessary in ISTE. In this way, ISTE processes are simpler and less labor intensive. The main drawback of ISTE processes is the use of higher volumes of methanol than conventional processes. Although, after reaction, the excess of methanol is recovered to be reused in a next batch, the cost involved in such process could be an obstacle to obtain a profitable method. Thus, costs and energy consumption of both processes (ISTE and conventional) should be analyzed and compared to determine, which is the most suitable method to obtain biodiesel from different raw matters. The reduction of energy consumption in the whole process, such as the reduction of reaction time, could contribute for an economic balance. Alternatives to reduce the amount of methanol in ISTE processes, such as the use of co-solvents, are being investigated with oil seeds [37] and could be studied to obtain biodiesel from yeasts.

5. Conclusion

We optimized an ISTE process to obtain yeast biodiesel, which consisted in heating the dry yeast biomass for 3 h at 70°C in the presence of methanol (methanol–biomass ratio = 60) and 0.4 M H_2SO_4 as the catalyst. The process was equally efficient to obtain biodiesel from *R. graminis* S1/S2 (a basidiomycetous yeast), and *C. glaebosa* 12D, which belongs to the phylum Ascomycota. Compared with the two-step process used for the industrial synthesis of biodiesel from oil seeds, the ISTE method offers the advantage of reducing the time and number of steps of the reaction, thereby resulting in a faster and cheaper process.

Conflict of interest

The authors report no relationships that could be construed as a conflict of interest.

Financial support

This work was supported by the Agencia Nacional de Investigación e Innovación (ANII), the Comisión Sectorial de Investigación Científica (CSIC Uruguay), and the Programa de Desarrollo de las Ciencias Básicas (PDECIBA).

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