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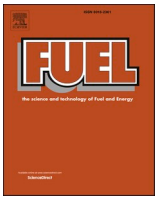

Full Length Article

One-pot fungal biomass-to-biodiesel process: Influence of the molar ratio and the concentration of acid heterogenous catalyst on reaction yield and costs

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ABSTRACT

This work evaluates a microbial-based biodiesel production process through simultaneous esterification and transesterification of the lipid-rich fungal biomass using ethanol as both extractor and acyl acceptor. Two of the most influential parameters were evaluated, the molar ratio of ethanol to the oil, and the concentration of a heterogeneous acid catalyst (H_3PMo/Al_2O_3), with responses in terms of conversion to ethyl esters and an estimated cost of the process variables. Fungal biomass of *Mucor circinelloides* was cultivated in sugarcane molasses media and the obtained oleaginous cells were used as the source of acylglycerols and free fatty acids for the trans/esterification reactions in a pressurized stainless-steel reactor at 200 °C for 6 h. Our results demonstrate that the effects of the two factors analyzed were significant, with an indication that increases in the molar ratio and catalyst favor the reaction yield. In the reaction system, a molar ratio of 120:1 (ethanol: oil) and 15 wt% of catalyst yielded 96.6% of ester content, which meets the minimum limit established by the international standards. Production costs were estimated in function of ethanol and catalyst price and indicated the selected parameters reflected the adequate configuration to reach the stablished minimum ester content.

1. Introduction

The use of microbial oils in the development of biorefineries has been a promising alternative to saponifiable lipids derived from vegetable crops and animal residues [1]. Oils derived from microorganisms, as microalgae, bacteria, and fungi, are, at a large extent, unaffected by seasonal effects and by climate, usually achieving high productivity yields [2]. Some species of fungi have been studied for decades as an alternative source of acylglycerols with characteristics close to those observed in vegetable oils [3]. Additionally, filamentous oleaginous fungi have the operational advantage of providing easier harvesting when compared to other unicellular microorganisms and have been demonstrated to be able to assimilate a wide range of carbon sources, and to be tolerant to low levels of inhibitors, as phenols and furfural [4].

The development of sustainable practices involving the use of fungi are largely related to their use in the design of fungal biorefineries, in order to, at a future extent, reduce waste generation and emission of polluting gases caused by conventional fossil-fuel based processes [5]. Most of these processes rely on agro-industrial residues, composed by, among others, vegetable biomass, which can be decomposed to form building blocks for marketable molecules, as fuels and products [6]. In this context, filamentous fungi emerge as a promising resource in the development of new sustainable products, among the species *Mucor circinelloides* stands as a producer of compounds that can be converted to products of industrial and commercial interests. *M. circinelloides* has been proven to grow efficiently in different agro-industrial residues [3], which allows their exploitation in the biodiesel production process. In a larger extent, *M. circinelloides* and other fungal species are model microorganisms to the development of biorefineries based on filamentous fungi, which are receiving increasing attention worldwide.

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Over the last years there has been an increase in the interest of providing feasible conversion routes of fungal lipids to produce biodiesel through trans/esterification reactions [3,7]. Some of the common operational bottlenecks described in the conversion of fungal lipids to biodiesel include the high acidity of the oil, in terms of high content of free fatty acids [8], and operational challenges in the extraction and purification of the lipids prior to the reaction [9]. In this sense, two common constraints that have been guiding some of the studies in the area, which are meant to address such concerns, include the use of catalysts that are largely unaffected by the high acidity of the oil [10], and the use of in-situ conversion technologies, i.e., based on simultaneous oil extraction and conversion in the presence of an acylating agent and a solvent, and often, a co-solvent.

The choice of an appropriate catalyst for an in-situ conversion approach has been widely explored and reported in the literature, of which, the use of enzymatic and acid catalysts prevails due to the impracticality associated with the use of acyl oxides and alkali substances [11]. Among these, the use of heterogenous materials allows recycling and reuse in sequential batches, or even the application of continuous production systems [12]. In particular, heterogeneous acid catalysts, such as the ones used in this work, composed of heteropoly acids supported onto a mineral oxide matrix, have been described to efficiently convert free fatty acid-rich feedstock to biodiesel in the presence of acyl acceptors as methanol and ethanol [10]. These acid catalysts are known to catalyze both esterification of free fatty acids and the transesterification of acylglycerols to produce alkyl esters [13,14].

In these lines, the development of simultaneous esterification and transesterification of fungal lipids are also dependent on reaction conditions that allow the extraction of the intracellular lipids to the reaction medium containing the acyl acceptor and the catalyst. Some previous reports of our group described the use of ethanol as an efficient lipid extractor [7,8,15], simultaneously acting as the acyl acceptor in the reaction. Ethanol molecules are also known to be miscible in water, which is a desirable trait in the selection of the solvent for this reaction in order to avoid costs associated with the biomass drying process. In addition, the production of ethyl-biodiesel helps to reinforce the concept of a renewable fuel, as not only the lipid source is coming from microbial cultures, but also the ethanol, which is produced, almost entirely at the commercial scale, from renewable plant sources, as maize and sugarcane [16].

This work, thus, presents a one-pot approach to produce microbial-based biodiesel through simultaneous esterification and transesterification of the lipid-rich fungal biomass using ethanol as both extractor and acyl acceptor. We present an in-depth study on two of the most influential parameters of this reaction, i.e., the molar ratio of ethanol to the oil, and the concentration of the heterogeneous acid catalyst, with responses in terms of conversion to ethyl esters and an estimated cost of the process variables.

2. Material and methods

2.1. Materials

Commercial Molasses from sugarcane was provided by Mellaço de Cana (Saltinho, Brazil), having the characteristics described by Bento et al. (2020). The main components in the molasses samples consist in sucrose (39.9%), fructose (7.5%), glucose (5.5%), potassium (25570 mg/kg), magnesium (4150 mg/kg) and other minerals in minor quantity [8]. 12-molybdophosphoric acid ($H_3PMo_{12}O_{40}$) was purchased from Sigma-Aldrich and the catalyst support (aluminum oxide, Al_2O_3) was provided by Alcoa Aluminum Company S. A. (Santo André, SP, Brazil). All the other chemical reagents were of analytical grade or higher purification.

2.2. Cell growth and analysis of biochemical parameters

The fungal strain of *Mucor circinelloides* f. *griseo-cyanus* URM 4182 obtained from the mycology collection (URM) from the Federal University of Pernambuco (Recife, Brazil) was used in all experiments. Mycelia was previously grown on PDA (Potato dextrose agar) at 30 °C for 72 h for sporulation and storage. The cultivation of the oleaginous cells was initiated by the addition of 1×10^5 spores mL^{-1} to a bioreactor model BioFlo/CelliGen® 115 (Eppendorf, CT), containing as medium diluted sugarcane molasses (40 g L^{-1} of total sugars content) with addition of minor synthetic nutrients as previously selected by Bento et al. [8]. The cultivations were carried out aerobically (air supply set to 1.5 vvm – volume of air per volume of medium - and agitation at 250 rpm) at 26 °C for 120 h. pH was adjusted and automatically maintained at 4.5 by a pH electrode sensor (Ingold, gel filled, Mettler Toledo, Greifensee, Switzerland).

The calculation of the biochemical parameters based on sugar consumption was based on data acquired from a High-Performance Liquid Chromatography (HPLC) system (Agilent 1200, Santa Clara, CA) equipped with a Refractive Index Detector (RID) operating at 35 °C and exchange resin column Bio-Rad Aminex HPX-87H (Bio-Rad Laboratories, Hercules, CA) operating at 18 °C. The mobile phase was H_2SO_4 solution (0.005 mol L^{-1}) at the flow rate of 0.5 mL·min^{-1}.

2.3. Lipid characterization

All the values involved in the calculation of biochemical parameters were estimated based on the dry biomass weight. Lipid extraction from the oleaginous microbial biomass was carried out through a microwave-assisted extraction method with ethanol in a microwave reactor (Model Discover/University-Wave - Cem Corporation, NC, USA) [17]. Fatty acid composition was determined as fatty acid methyl ester (FAME) by gas chromatography according to American Oil Chemists' Society (AOCS) official method Ce 1–62.

2.4. Direct transesterification reaction

FAEE were produced via direct transesterification of wet biomass using heterogeneous acid catalyst and carried out in a one-step process integrating extraction/reaction of the intracellular lipids to ethyl ester (one-pot reaction), using anhydrous ethanol as both solvent for lipid extraction and acyl acceptor [17]. The catalyst was prepared by wet incipient impregnation of H_3PMo in alumina as described elsewhere [10].

To a 100 mL pressurized stainless-steel reactor (Parr Series 5500, Parr Instruments, Molline, IL) wet microbial biomass (containing 2.0 g, lipid, 30% water content) was added with the required amount of anhydrous ethanol and the prepared heterogenous acid catalyst. The reaction vessels were heated to 200 °C for 6 h and vigorously agitated by mechanic stirring at 300 rpm. At the final of the reaction, the resulting mixture was cooled and filtered. The products were purified by dry washing using Chamotte® clay, was rotatory vacuum-evaporated at 80 °C [18], and the products were dried over a small amount of anhydrous sodium sulfate. The purified product was analyzed by NMR^1H in a Mercury 300 MHz Varian spectrometer using deuterium-chloroform as solvent and the ethyl esters content (wt. %) was calculated based on the methodology described by Paiva et al. [19], which is based in the relation of the integration of the fourth peak signal of the quartet (4.08 ppm) and the whole ethoxy-carbons hydrogen area in the region from 4.05 to 4.20 ppm since this forth peak is in a region where crossover does not occur and can be successfully assigned to ethyl esters.

Finally, FTIR (Spectrum GX FTIR spectrometer, Perkin Elmer, MA, USA) was used to qualitatively determine the presence of residual lipids in the biomass after the extraction, using the in natura oil-rich biomass as control. The FTIR analysis were carried at using samples prepared using KBr and read within the range of 4000–400 cm^{-1} wavenumber

[20].

2.5. Direct transesterification: Parameters evaluation

The influence of molar ratio (ethanol:oil) and catalyst concentration (wt.%) were evaluated by Response Surface Methodology applying a center composition factorial design, as described in Table 1. Results were analyzed in function of ester content (wt.%) using Statistica 12.5 softwere (Statsoft). The values present in Table 1 are based on indications described by Da Conceição et al. [8], who carried out an evaluation of the use of a heteropolyacid supported onto niobium oxide as a catalyst in reactions using lipid feedstocks containing high levels of free fatty acids, including fungal single cell oil.

3. Results and discussion

3.1. Oleaginous fungal biomass production

The lipid accumulation by this fungal strain has been thoroughly explored in other studies [2,3] having been demonstrated that the use of molasses, given the appropriate constraints of C:N ratio, can yield high lipid productivities [8]. In fact, as it can be seen from Table 2, for each gram of sugar present in the substrate, approximately 0.3 g of biomass was accumulated, of which, approximately 30% is composed by lipids. The fatty acid distribution of these lipids, as it is also described in Table 2, shows a balance between saturated and unsaturated fatty acids, which, according to represent a sum of the high stability to oxidation characteristic to the saturated fatty acids with the appropriate viscosity and kinematic properties of the unsaturated carbon chains. The high acidity value of the extracted lipids (38.22 mg KOH g^{-1}) indicates the need of applying acid or enzymatic catalysts for further modifications to avoid the formation of soap. These values are in accordance to a previous characterization of microbial oil produced by *M. circinelloides*, which indicated acidity of 38.8 mg KOH g^{-1} oil [7].

The industrial application of microbial oils can be limited by the high costs in the fermentation processes to obtain oilseed biomass. The carbon source used for SCO cultivation is responsible for a large part of production costs. Usually for the cultivation of fungi with concomitant lipid accumulation, conventional carbon sources, such as glucose, are used, however, the introduction of alternative substrates appears as an interesting option to reduce process costs [21].

3.2. Evaluation of molar ratio and catalyst concentration influence on the reaction

The influence of two of the most influential parameters on direct simultaneous trans/esterification reactions [22], molar ratio and catalyst concentration, were evaluated by a complete factorial design with triplicate at the central point. The experimental matrix and the results obtained regarding the ester content in the final product are shown in Table 3. From the results, we can observe that the H_3PMo/Al_2O_3 catalyst was able to provide high esters in all analyzed reactions (91.9–96.6%), having the highest value being achieved by applying molar ratio (alcohol:oil) of 120:1 and catalytic loading of 15 wt%. The reaction that provided the lowest ester content was carried out at a molar ratio of 60:1 and 5% of catalyst, these results indicate that both the molar ratio and the concentration of catalyst have a positive influence on the formation of esters.

Table 1
Parameters of factorial design applied for direct transesterification optimization.

Variable	Level		
	−1	0	+1
Molar ratio (ethanol:oil)	60	90	120
Catalyst concentration (wt. %)	5	10	15

Table 2
Biochemical parameters involved in the growth of *M. circinelloides* URM 4182 in a medium composed by sugarcane molasses supplemented with minor nutrients.

Biochemical parameters	Values
X - Biomass concentration (g L^{-1})	11.25 ± 0.55
Q_x - Biomass productivity (g L^{-1} day^{-1})	2.25 ± 0.11
$Y_{x/s}$ - Specific yield of biomass (g biomass g^{-1} substrate)	0.30 ± 0.02
Substrate (total sugar) consumption (g L^{-1})	37.03 ± 0.98
Substrate (total sugar) consumption (%)	91.12 ± 0.61
P - Lipid concentration (g L^{-1})	3.31 ± 0.16
Lipid content (wt.%)	29.4 ± 0.8
Q_p - Lipid productivity (g L^{-1} day^{-1})	0.66 ± 0.03
$Y_{p/x}$ - Specific yield of lipid (g lipid g^{-1} biomass)	0.29 ± 0.08
$Y_{p/s}$ - Product yield (g lipid g^{-1} substrate)	0.09 ± 0.01
Lipid molar weight (g mol^{-1})	812.18 ± 4.24
Lipid acidity value (mg KOH g^{-1})	38.22 ± 1.35
Fatty acid composition of lipids	
C16:0 (wt.%)	28.29 ± 1.02
C18:1 (wt.%)	21.01 ± 0.97
C18:3 (wt.%)	17.22 ± 0.63
C18:2 (wt.%)	10.94 ± 0.58
C10:0 (wt.%)	6.55 ± 0.40
C14:0 (wt.%)	5.21 ± 0.22
Others (wt.%)	10.78 ± 1.25
Saturated fatty acids (SFA, wt.%)	52.17 ± 1.61
Monounsaturated fatty acids (MUFA, wt.%)	24.01 ± 1.09
Polyunsaturated fatty acids (PUFA, wt.%)	28.16 ± 0.87

*Confidence intervals represent the standard deviation of triplicate runs.

Table 3
Response surface experimental matrix considering molar ratio and catalyst amount as factors and content of ethyl esters in the product as response.

Run	Factors				Response Values
	Molar Ratio X_1		Catalyst (wt. %) X_2		Ester Content (%)
	Codified variable	Value	Codified variable	Value	
1	+	120:1	+	15	96.6
2	+	120:1	−	5	93.7
3	−	60:1	+	15	93.2
4	−	60:1	−	5	91.9
5	0	90:1	0	10	94.5
6	0	90:1	0	10	94.7
7	0	90:1	0	10	94.8
8	+	120:1	0	10	95.6
9	−	60:1	0	10	92.8
10	0	90:1	+	15	95.6
11	0	90:1	−	5	93.4

The effects of the analyzed independent variables allow to verify that the linear term referring to variable X_1 (Molar ratio) was the one that had the greatest influence due to the content of esters, followed by the linear term referring to variable X_2 (% catalyst). The positive effect of these factors indicates that there is an upward trend in ester content employing higher molar ratios and catalyst concentration. From the results, it is also observable that the significant quadratic terms with negative effects indicate that the model can be maximized. The interaction between the factors, which is also significant, had a lower positive effect when compared to the isolated linear terms. However, this result may indicate that a higher content of ethanol can promote an increase the access of the oil to the active sites of the catalyst. The Pareto chart of the analysis detailing the effects can be seen in the Supplementary Material.

The positive influence of the increase in the molar ratio, that is, a greater proportion of ethanol in the reaction medium, may be related to the fact that ethanol acts simultaneously as an extraction solvent and a reagent in the simultaneous esterification and transesterification reactions. In this way, a greater amount of ethanol in the medium

increases its availability, since it is also consumed during the reaction, favoring the mass transfer phenomena involved and facilitating the performance and access of the catalyst on the substrate.

Ethanol, in fact, has been described to be an effective lipid extractor for *M. circinelloides* oleaginous biomass, as described elsewhere [17]. In this sense, we assume that the reaction kinetics and the associated mechanism are related to the sequential steps of cell rupture and lipid diffusion into the solvent medium, followed by the simultaneous transesterification of acylglycerols and esterification of free fatty acids with ethanol catalyzed by H_3PMo/Al_2O_3, based on observations from the literature [7,9,17,20]. Fig. 1 depicts the representation of the proposed mechanism, considering ethanol molecules as the blue triangles and the blue background, cell wall as the green circles, lipid droplets as the yellow circles, and the H_3PMo/Al_2O_3 catalyst as the dashed rectangles.

The discussion of the influence of the factors and the proposed mechanism is also in accordance with the analysis of variance (ANOVA) of the data analyzed in this work. Table 4 presents the ANOVA of such factors, both linear (X_1) and quadratic (X_2^2), and the interaction between the factors ($X_1 \times X_2$). A brief analysis from the ANOVA considering a significance level of 95% indicates the possibility of the rejection of the random variability of the null hypothesis for the evaluated terms on the regression model. In fact, considering these data, the statistical model to predict the response as conversion yield in terms of content of esters in the product has a high correlation coefficient value of $R^2 = 0.99566$. Eq. 1 represents the model using these codified values and considering Y as the content of esters (%) in the product.

$$Y = 94.705 + 1.333 \times X_1 - 0.563 \times X_1^2 + 1.067 \times X_2 - 0.263 \times X_2^2 + 0.400 \times X_1 \times X_2 \quad (1)$$

From the regression model, a response surface was generated to predict the optimized values of the content of esters in the final product considering the molar ratio and the concentration of the H_3PMo/Al_2O_3 catalyst (Supplementary Material). The experimental results that yielded the highest content of esters in the product phase was attained using a molar ratio of 120:1 and a catalyst concentration of 15 wt%, resulting in 96.6% esters in the product mixture. From a qualitative point of view, we can still observe that with a further increase of the molar ratio and the weight contribution of the catalyst would improve even further the response factor. Lastly, the experimental results were analyzed in terms of the lack of fit on the regression model using a chart of the predicted values versus the observed values (Supplementary Material), which, again, dictates the good fit of the model constructed from the experimental observations.

Table 4
Analysis of Variance (ANOVA) of the model considering linear and quadratic factors, and their interaction. Where X_1 = Molar ratio (ethanol:oil) and X_2 = catalyst concentration.

Factor	Sum of Squares (SS)	Degree of freedom (df)	Mean Squares (MS)	F	p-value
Molar Ratio (X_1)	10.66667	1	10.66667	630.7054	0.000002
Molar Ratio^2 (X_1^2)	0.80344	1	0.80344	47.5062	0.000984
Catalyst (X_2)	6.82667	1	6.82667	403.6515	0.000006
Catalyst^2 (X_2^2)	0.17544	1	0.17544	10.3734	0.023445
($X_1 \times X_2$)	0.64000	1	0.64000	37.8423	0.001651
Error	0.08456	5	0.01691		
Total SS	19.48727	10			

Considering that the model predicted that an increase in the catalyst amount demonstrates a positive output on the response, and that the molar ratio of 120:1 when applied concomitantly with a catalyst amount of 20% yielded a content of esters of 96.7%, we pursued a series of screening experiments to evaluate the effect of the range of catalyst from 10 to 30 wt%. The results, as depicted in Fig. 2a, show that there is not a strong influence of the catalyst amount on this range at the molar ratio of 120:1, and that the catalyst applied at any concentration greater than 15 wt% on these conditions should suffice the minimum conversion yield of 96.5% representative of the regulatory norms of esters of biodiesel quality. Similarly, Fig. 2b indicate a variation in the molar ratio (ethanol:oil) considering the amount of 15 wt% and can be seen no significant difference in an increase in the molar ratio over 120. As is detailed discussed in the section 3.4 of this study, the increase of ethanol and catalyst amounts may promote an undesirable response elevating the process costs and so, should be deeper evaluated. Thereby, the most appropriated reaction conditions seems to be 15% of catalyst and molar ratio of 120:1 (ethanol:oil).

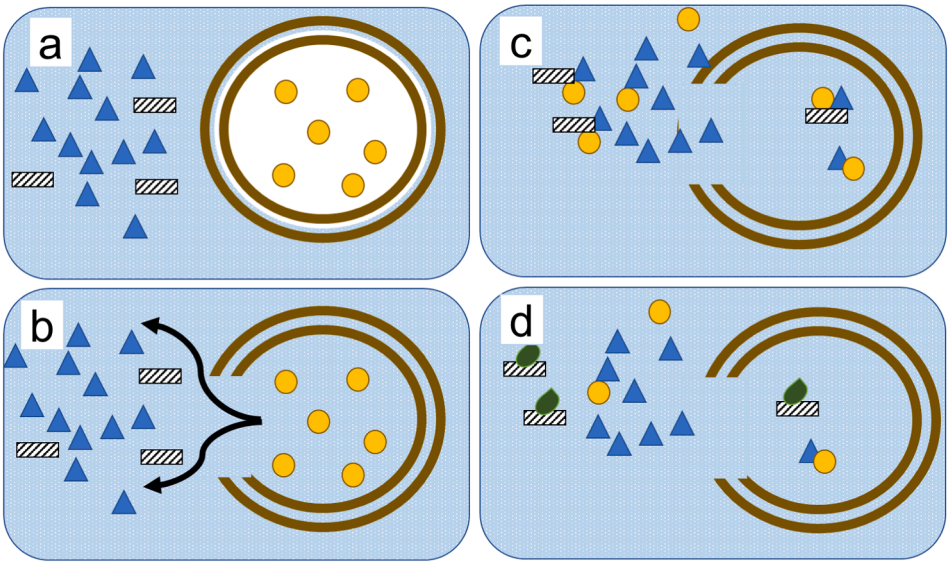

Fig. 1. Proposed mechanism for one-pot biomass-to-biodiesel process: a. fungal cell suspension on the ethanol phase, b. cell wall lysis and diffusion of intracellular lipids to the ethanol phase, c. placement of the reactants for the esterification (free fatty acids and ethanol) and transesterification (tri-, di-, and monoacylglycerols and ethanol) onto the catalytic sites of H_3PMo/Al_2O_3, and d. production of ethyl esters and by-products (water or glycerol) and release from the catalytic site.

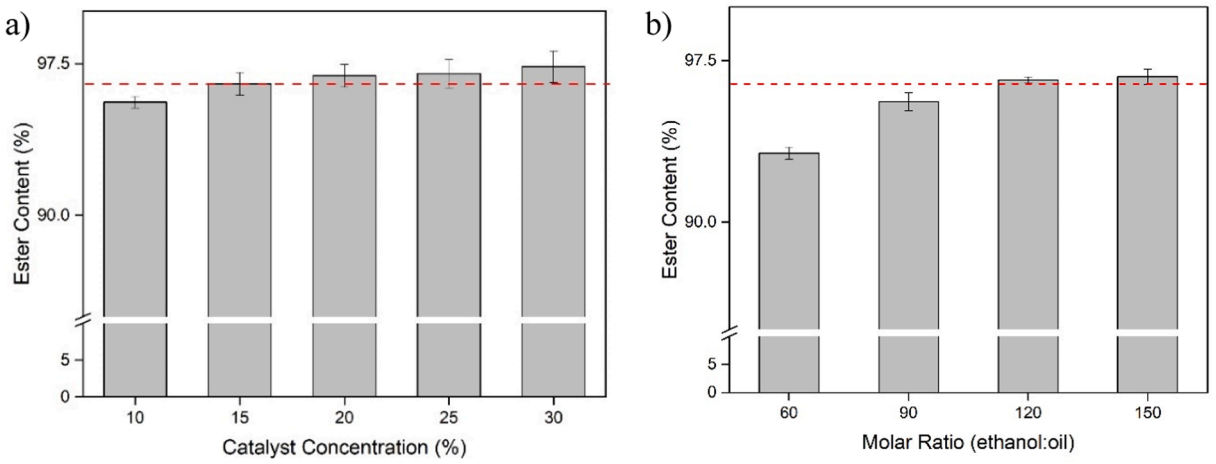

Fig. 2. a) Effect of the concentration of the H_3PMo/Al_2O_3 catalyst at a molar ratio of 120:1 on the esters content; b) Effect of the molar ratio using 15 wt% of catalyst (relative to oil amount) on the esters content; highlighting the minimum threshold of 96.5% of ester content as the red dashed line. (Confidence intervals represent the standard deviation of duplicate runs). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.3. Residual biomass characterization

Direct transesterification may lead to mass transfer limitation and imply in non-extracted lipids residually in the biomass. Therefore, an analytical technique that allows monitoring residual lipids in the biomass would be unavoidable. Here, biomass *in natura* and after reaction was characterized by FTIR spectroscopy. Fig. 3 shows both obtained spectra.

It can be seen in the Fig. 3 the disappearance of the 3005 and 1740 cm^{-1} bands related to = C–H stretching of unsaturated fatty acids and C = O stretching of esters, respectively. There is also a reduction in the bands present at 2920 cm^{-1}, 2855 cm^{-1} related to the stretching of the C – H bond in $–CH_3$ and $–CH_2$ characteristics of fatty acids and fatty acid esters, as also seen in CH_2 rocking band at 720 cm^{-1} [23]. The large band at 3500 cm^{-1} related to hydroxyl groups (OH) may be associated to the presence of water or adsorbed glycerol in the biomass [20].

These results indicate that mostly of the lipids were extracted from the fungal biomass in the direct reaction process, showing the efficiency of the method. However, the signs in the FTIR spectrum indicated the presence of residual lipids in the biomass after the reaction. In order to elucidate the extraction process efficiency, the recovered reacted biomass was submitted to an exhaustively extraction using ethanol under microwave irradiation (60 °C, 3 cycles of 60 min) and the residual lipids were quantified by gravimetry. The analysis showed 2.4 wt% of the initial lipid content in the reaction was still present in the recovered biomass, corresponding to an extraction efficiency of 97.6% in the simultaneous reaction and extraction process.

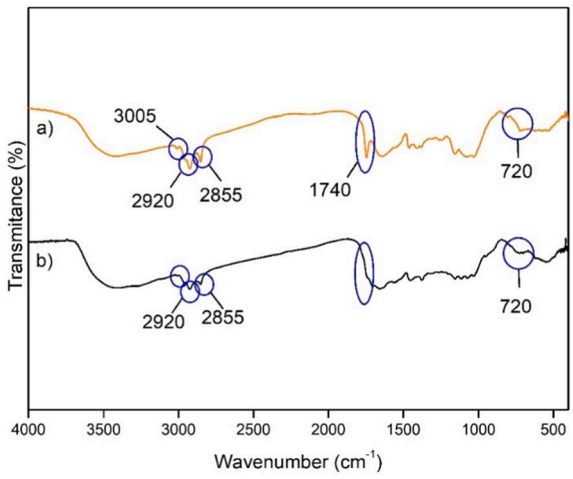

Fig. 3. FTIR spectra of a) *In natura* fungal biomass and b) Fungal biomass after one-pot reaction.

3.4. Economic constraints and perspectives

One of the greatest challenges in the implementation of efficient and optimized processes to produce microbial-based fuels is the scale [24]. Most processes and examples in the literature deal with small volume of production, due to constraints of biomass accumulation and laboratory equipment. In this sense, we believe that the findings of this article may aid a fine-tuning process of the variables used in the biomass-to-biodiesel reaction system. Many of the studies reported are either demonstrative, on a sense that the work effort serves to prove the technical feasibility of a given reaction conditions, the results described in this work could help, thus, to unlock not only mechanistic insights to the process as a whole, but also to provide indications of what process variables could be factored in future scale-up studies and applications.

A preliminary operating cost analysis based on the two factors involved in the reaction of the study was developed given the considerations for estimating the cost of production of a commercial heterogeneous catalyst and the use of ethanol. We did not evaluate herein the energy costs associated with the upstream and downstream of the reaction, as these were kept constant throughout the assays. The costs of a Ni/Al_2O_3 catalyst [25] would be in the range of USD 20.59 lb^{-1}, which, assuming a fixed cost of the processing of the Al_2O_3 to be 70% of the total cost, and the remaining 30% associated with the adsorbed catalytic material, and assuming that ratio of the cost of H_3PMo over Ni is approximately 2.33 based on commercial values of the two reagents, the cost of the H_3PMo/Al_2O_3 could be roughly estimated to be in the range of USD 28.81 lb^{-1}. The cost of anhydrous ethanol was assumed to be USD 0.45 L^{-1}. Based on an extrapolation of data available in the literature [10] on the reusability of a H_3PMo/Al_2O_3, we assumed that the catalyst could be recycled for five consecutive reaction cycles. A conservative approach was taken into consideration of the use of ethanol, assuming it would only be used once. Therefore, Eqs. (2) and (3) were used to estimate the reaction associated cost of the catalyst and ethanol, assuming the cost of 1 kg of oil processed as a basis for estimation. The factors "Molar Ratio" and "Catal (wt.%)" are those found in the experimental design, MW_{oil} and MW_{EtOH} are the molar weights of the oil and anhydrous ethanol, respectively, in mol g^{-1}, ρ_{EtOH} is the specific gravity of ethanol in kg L^{-1} (0.78 kg L^{-1}), and $ denote the USD sign. The total processing cost was assumed to be the result of Eq. X plus Eq. Y given the

experimental planning.

$$EtOH\ \ cost\ \ \left(USD\ \ kg^{-1} oil\ \ processed\right) = \frac{1\ \ kg_{oil}}{MW_{oil}} \times Molar\ \ Ratio \times \frac{MW_{EtOH}}{\rho_{EtOH}} \times \frac{\$\ \ 0.45}{L} \quad (2)$$

$$H_3PMo/Al_2O_3 cost\ \ \left(USD\ \ kg^{-1} oil\ \ processed\right) = Catal\ \ (wt.\%) \times 1\ \ kg_{oil} \times \frac{1}{5} \times \frac{\$46.17}{kg} \quad (3)$$

Table 5 summarizes the estimated cost for the experimental planning, and Fig. 4 depicts the response surface generated for the data associated with these estimated costs. As it can be seen from Fig. 4, an increase in the molar ratio and in the amount of ethanol is, unsurprisingly, associated with an increase in the costs of the reaction. Given the experimental constraints of producing an ester mixture with a product yield of at least 96.5%, it can be roughly estimated from a visual analysis, that there is an unnecessary increase in the costs with an increase in the catalyst amount of over 15 wt% and molar ratio of over 120:1 (ethanol to oil). Therefore, in these lines, one should take into consideration that biomass direct transesterification should be optimized aiming to guarantee the minimum ester content stablished by international standards and the minimum processing costs.

Over the past years, the scientific community has advanced significantly in the field of one-pot reactions to trans/esterification reactions to produce either methyl or ethyl esters of biodiesel quality. For instance, Rasmey et al. [26], who reported the transesterification of *Fusarium solani* biomass, demonstrated a one-pot conversion yield of 97.63% for a reaction medium containing 500 mg of dry fungal mycelium suspended in 50 mL of a mixture of methanol, hydrochloric acid, and chloroform (10:1:1 v/v/v), which was heated for 1 h at 90 °C at a thermostatic bath. Sitepu et al. [27] also reported a methyl-based conversion catalyzed by NaOH, achieving 90% of methyl esters in the product mixture using *Mucor plumbeus* biomass as the lipid source for the reaction. At a lower temperature, yet through an interesting approach, Kakkad et al. [28] reported the preparation of methyl esters using *Aspergillus candidus* biomass through a reaction catalyzed by H_2SO_4 with the presence of chloroform as a solvent using sonication as a simultaneous physical treatment in order to ease the cell rupture process, reaching a product yield of approximately 70%. There are fewer studies reporting the use of heterogeneous catalysis for similar purposes. Among some of the recent reports, Vasiliadou et al. [29] described the reaction of two fungal strains, *Trametes versicolor* and *Ganoderma lucidum*, as feedstock for a methanol-based conversion using a small autoclave-type reactor catalyzed by Zr-SBA. Bento et al. [8] reported the use of H_3PMo/Al_2O_3 as catalyst for the conversion of *M. circinelloides* biomass, reaching 96.5% of ester content. In this sense, the results demonstrated herein are promising and could be coupled with other reaction designs using ultrasonics or critical conditions, considering the high yields recently reported by Martínez et al. [30 31] and Pascoal et al. [32].

Table 5
Estimated costs of the reaction in USD per kg of oil processed.

Run	Factors				Response Values
	Molar Ratio X_1		Catalyst (wt. %) X_2		Cost (USD per kg of oil processed)
	Codified variable	Value	Codified variable	Value	
1	+	120:1	+	15	5.31
2	+	120:1	–	5	4.38
3	–	60:1	+	15	3.35
4	–	60:1	–	5	2.42
5	0	90:1	0	10	3.86
6	0	90:1	0	10	3.86
7	0	90:1	0	10	3.86
8	+	120:1	0	10	4.84
9	–	60:1	0	10	2.88
10	0	90:1	+	15	5.31
11	0	90:1	–	5	4.38

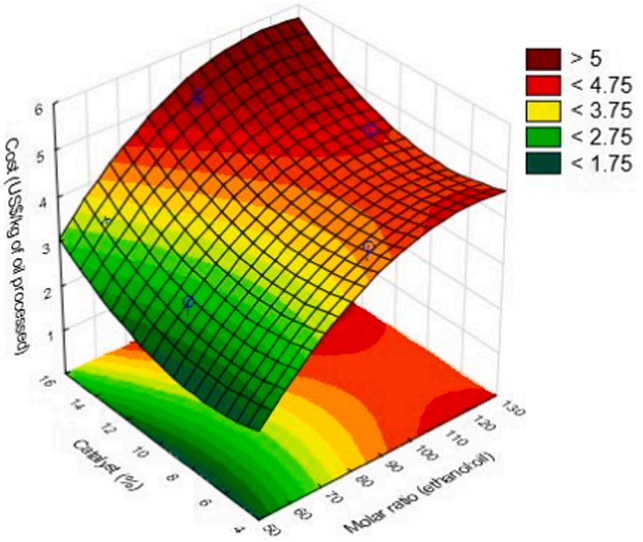

Fig. 4. Response surface for the response of content of cost (USD kg^{-1} oil processed) in the products considering catalyst concentration (wt.%) and lipids: ethanol molar ratio.

4. Conclusion

This work presented an evaluation of some of the operating conditions involved in a one-pot synthesis of biodiesel from wet fungal biomass using heterogeneous acid catalysis. The study screened the influence of the concentration of the catalyst in regard to the amount of lipids present and the molar ratio of ethanol:lipids. The results demonstrate that increasing both parameters lead to higher conversion rates under the fixed conditions of temperature and time. Nonetheless, according to our preliminary economic assessment, results that meet the international norms of ester content in the product mixture can be obtained using a 120:1 M ratio of ethanol to lipids at a catalyst ratio of 15 wt%. Above these values, the costs associated with the production increase unnecessarily. To a greater extent, within the extended possibilities of biorefinery models, and particularly those with a core in the conversion of oleaginous biomass to liquid fuels, as biodiesel, this work becomes a fundamental part in the overall design process. Even though the model of a sustainable biorefinery is not a fixed concept, depending on, among other factors, geopolitical factors that affect the availability of the acylating agent for the reaction, subsidies, and availability of the feedstocks used both in the fungal growth process, as well as those used for the preparation of the catalyst, the indication of the significance of the process variables is crucial for evaluating the technical and economic feasibility of one-pot-like processes within the design of integrated biorefineries.

CRediT authorship contribution statement

Heitor B.S. Bento: Conceptualization, Visualization, Formal analysis, Investigation, Data curation, Writing - original draft. **Cristiano E. R. Reis:** Validation, Visualization, Writing - original draft. **Pietro G. Cunha:** Investigation, Formal analysis, Data curation, Writing - original draft. **Ana K.F. Carvalho:** Methodology, Investigation, Validation, Visualization, Writing - original draft. **Heizir F. De Castro:** Conceptualization, Resources, Supervision, Project administration, Funding acquisition, Writing - original draft.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fuel.2021.120968.

References

[1] Chew KW, Yap JY, Show PL, Suan NH, Juan JC, Ling TC, et al. Microalgae biorefinery: High value products perspectives. Bioresour Technol 2017;229:53–62.
[2] Ratledge C, Cohen Z. Microbial and algal oils: Do they have a future for biodiesel or as commodity oils? Lipid Technology 2008;20(7):155–60.
[3] Rodrigues Reis CE, Bento HBS, Carvalho AKF, Rajendran A, Hu B, De Castro HF. Critical applications of *Mucor circinelloides* within a biorefinery context. Crit Rev Biotechnol 2019;39(4):555–70.
[4] Reis CER, Zhang J, Hu B. Lipid Accumulation by Pelletized Culture of *Mucor circinelloides* on Corn Stover Hydrolysate. Appl Biochem Biotechnol 2014;174(1):411–23.
[5] Troiano D, Orsat V, Dumont MJ. Status of filamentous fungi in integrated biorefineries. Renew Sustain Energy Rev 2020;117:109472.
[6] Fermoso FG, Serrano A, Alonso-Fariñas B, Fernández-Bolaños J, Borja R, Rodríguez-Gutiérrez G. Valuable Compound Extraction, Anaerobic Digestion, and Composting: A Leading Biorefinery Approach for Agricultural Wastes. J Agric Food Chem 2018;66(32):8451–68.
[7] Carvalho AKF, Rivaldi JD, Barbosa JC, de Castro HF. Biosynthesis, characterization and enzymatic transesterification of single cell oil of *Mucor circinelloides* – A sustainable pathway for biofuel production. Bioresour Technol 2015;181:47–53.
[8] Bento HBS, Carvalho AKF, Reis CER, De Castro HF. Single cell oil production and modification for fuel and food applications: Assessing the potential of sugarcane molasses as culture medium for filamentous fungus. Ind Crops Prod 2020;145:112141.
[9] Reis CER, Valle GF, Bento HBS, Carvalho AKF, Alves TM, de Castro HF. Sugarcane by-products within the biodiesel production chain: Vinasse and molasses as feedstock for oleaginous fungi and conversion to ethyl esters. Fuel 2020;277:118064.
[10] da Conceição LRV, Reis CER, de Lima R, Cortez DV, de Castro HF. Keggin-structure heteropolyacid supported on alumina to be used in trans/esterification of high-acid feedstocks. RSC Adv 2019;9(41):23450–8.
[11] Lam MK, Lee KT, Mohamed AR. Homogeneous, heterogeneous and enzymatic catalysis for transesterification of high free fatty acid oil (waste cooking oil) to biodiesel: A review. Biotechnol Adv 2010;28(4):500–18.
[12] Sheikh R, Choi M-S, Im J-S, Park Y-H. Study on the solid acid catalysts in biodiesel production from high acid value oil. J Ind Eng Chem 2013;19(4):1413–9.
[13] Caliman E, Dias JA, Dias SCL, Garcia FAC, de Macedo JL, Almeida LS. Preparation and characterization of $H_3PW_{12}O_{40}$ supported on niobia. Microporous Mesoporous Mater 2010;132(1):103–11.
[14] da Conceição LRV, Carneiro LM, Rivaldi JD, de Castro HF. Solid acid as catalyst for biodiesel production via simultaneous esterification and transesterification of macaw palm oil. Ind Crops Prod 2016;89:416–24.
[15] Zorn SMFE, Reis CER, Bento HBS, de Carvalho AKF, Silva MB, De Castro HF. In Situ Transesterification of Marine Microalgae Biomass via Heterogeneous Acid Catalysis. Bioenergy Res 2020;13(4):1260–8.
[16] Bouaid A, Martinez M, Aracil J. A comparative study of the production of ethyl esters from vegetable oils as a biodiesel fuel optimization by factorial design. Chem Eng J 2007;134(1):93–9.
[17] Carvalho AKF, Bento HBS, Izário Filho HJ, de Castro HF. Approaches to convert *Mucor circinelloides* lipid into biodiesel by enzymatic synthesis assisted by microwave irradiations. Renewable Energy 2018;125:747–54.
[18] Santos FD, da Conceição LRV, Ceron A, de Castro HF. Chamotte clay as potential low cost adsorbent to be used in the palm kernel biodiesel purification. Appl Clay Sci 2017;149:41–50.
[19] Paiva EJM, da Silva MLCP, Barboza JCS, de Oliveira PC, de Castro HF, Giordani DS. Non-edible babassu oil as a new source for energy production–a feasibility transesterification survey assisted by ultrasound. Ultrason Sonochem 2013;20(3):833–8.
[20] Carvalho AKF, Bento HBS, Reis CER, De Castro HF. Sustainable enzymatic approaches in a fungal lipid biorefinery based in sugarcane bagasse hydrolysate as carbon source. Bioresour Technol 2019;276:269–75.
[21] Koutinas AA, Yepez B, Kopsahelis N, Freire DMG, de Castro AM, Papanikolaou S, et al. Techno-economic evaluation of a complete bioprocess for 2,3-butanediol production from renewable resources. Bioresour Technol 2016;204:55–64.
[22] Verma P, Dwivedi G, Sharma MP. Comprehensive analysis on potential factors of ethanol in Karanja biodiesel production and its kinetic studies. Fuel 2017;188:586–94.
[23] Forfang K, Zimmermann B, Kosa G, Kohler A, Shapaval V. FTIR Spectroscopy for Evaluation and Monitoring of Lipid Extraction Efficiency for Oleaginous Fungi. PLoS ONE 2017;12(1):e0170611.
[24] Kamm B, Schönicke P, Hille C. Green biorefinery – Industrial implementation. Food Chem 2016;197:1341–5.
[25] Baddour FG, Snowden-Swan L, Super JD, Van Allsburg KM. Estimating Precommercial Heterogeneous Catalyst Price: A Simple Step-Based Method. Org Process Res Dev 2018;22(12):1599–605.
[26] Rasmey AHM, Tawfik MA, Abdel-Kareem MM. Direct transesterification of fatty acids produced by *Fusarium solani* for biodiesel production: effect of carbon and nitrogen on lipid accumulation in the fungal biomass. J Appl Microbiol 2020;128(4):1074–85.
[27] Sitepu EK, Jones DB, Zhang Z, Tang Y, Leterme SC, Heimann K, et al. Turbo thin film continuous flow production of biodiesel from fungal biomass. Bioresour Technol 2019;273:431–8.
[28] Kakkad H, Khot M, Zinjarde S, RaviKumar A. Biodiesel Production by Direct In Situ Transesterification of an Oleaginous Tropical Mangrove Fungus Grown on Untreated Agro-Residues and Evaluation of Its Fuel Properties. Bioenergy Res 2015;8(4):1788–99.
[29] Vasiliadou IA, Sánchez-Vázquez R, Molina R, Martínez F, Melero JA, Bautista LF, et al. Biological removal of pharmaceutical compounds using white-rot fungi with concomitant FAME production of the residual biomass. J Environ Manage 2016;180:228–37.
[30] Martínez A, Mijangos GE, Romero-Ibarra IC, Hernández-Altamirano R, Mena-Cervantes VY, Gutiérrez S. A novel green one-pot synthesis of biodiesel from *Ricinus communis* seeds by basic heterogeneous catalysis. J Cleaner Prod 2018;196:340–9.
[31] Martínez A, Mijangos GE, Romero-Ibarra IC, Hernández-Altamirano R. Mena-Cervantes VY. In-situ transesterification of *Jatropha curcas* L. seeds using homogeneous and heterogeneous basic catalysts. Fuel 2019;235:277–87.
[32] Pascoal CVP, Oliveira ALL, Figueiredo DD, Assunção JCC. Optimization and kinetic study of ultrasonic-mediated in situ transesterification for biodiesel production from the almonds of *Syagrus cearensis*. Renewable Energy 2020;147:1815–24.