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Direct Transformation of Fungal Biomass from Submerged Cultures into Biodiesel

Gemma Vicente,[†] L. Fernando Bautista,[†] Francisco J. Gutiérrez,[†] Rosalía Rodríguez,[†] Virginia Martínez,[†] Rosa A. Rodríguez-Frómeta,[‡] Rosa M. Ruiz-Vázquez,[‡] Santiago Torres-Martínez,[‡] and Victoriano Garre*[,‡]

[†]*Department of Chemical and Environmental Technology, ESCET, Universidad Rey Juan Carlos, C/Tulipán s/n, 28933 Móstoles, Madrid, Spain, and* [‡]*Departamento de Genética y Microbiología (Unidad Asociada al IQFR−CSIC), Facultad de Biología, Universidad de Murcia, 30071 Murcia, Spain*

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Diminishing fossil fuel reserves and the increase in their consumption indicate that strategies need to be developed to produce biofuels from renewable resources. Biodiesel offers advantages over other petroleum-derived fuel substitutes, because it is comparatively environmentally friendly and an excellent fuel for existing diesel engines. Biodiesel, which consists of fatty acid methyl esters (FAMEs), is usually obtained from plant oils. However, its extensive production from oil crops is not sustainable because of the impact this would have on food supply and the environment. Microbial oils are postulated as an alternative to plant oils, but not all oleaginous microorganisms have ideal lipid profiles for biodiesel production. On the other hand, lipid profiles could be modified by genetic engineering in some oleaginous microorganisms, such as the fungus *Mucor circinelloides*, which has powerful genetic tools. We show here that the biomass from submerged cultures of the oleaginous fungus *M. circinelloides* can be used to produce biodiesel by acid-catalyzed direct transformation, without previous extraction of the lipids. Direct transformation, which should mean a cost savings for biodiesel production, increased lipid extraction and demonstrated that structural lipids, in addition to energy storage lipids, can be transformed into FAMEs. Moreover, the analyzed properties of the *M. circinelloides*-derived biodiesel using three different catalysts (BF_3, H_2SO_4, and HCl) fulfilled the specifications established by the American standards and most of the European standard specifications.

1. Introduction

Society is facing an unprecedented situation with regard to the fundamental sources of its raw materials and energy. Petroleum, the fuel that has driven modern society for the last century, is showing signs of scarcity.[1,2] Many renewable fuel alternatives are under study,[3] but ethanol and biodiesel are already available in petrol stations. Biodiesel, which consists of fatty acid methyl esters (FAMEs), has many advantages, such as high energy density, great lubricity, fast biodegradation rate, and reduced emissions of sulfur, aromatic compounds, and particulate matter.[4] However, biodiesel adoption is complicated because it competes with the food industry for the main raw material input, plant oils, and the worldwide supply of plant oils is limited by land and water availability.[4,5] Moreover, a rapid expansion in biodiesel production capacity is being observed in not only developed countries, e.g., United States and European Union, but also developing countries. To meet the demand of this industry, oil sources other than crop oils should be quickly developed.[6] One way to increase world oil production that would cause a low ecosystem impact is to use lipids from oleaginous microorganisms (also called single-cell oils), which present many significant advantages over plants. Oleaginous microorganisms, such as yeasts, fungi, bacteria, and microalgae, can accumulate high levels of lipids[7−14] (Table 1) and do not require arable land, so that they do not compete with food production. More particularly, photosynthetic microalgae have attracted attention and investment because they capture carbon dioxide in lipids using sunlight. However, their growth in bioreactor systems is problematic because of the light supply requirement.[6,15] Oleaginous yeasts and fungi have also been considered as potential oil sources for biodiesel production because they accumulate large amounts of lipids. Among these microorganisms, particular attention has been dedicated to various oleaginous zygomycetes species, such as *Mortierella isabelina* and *Cunninghamella*

*To whom correspondence should be addressed: Departamento de Genética y Microbiología, Facultad de Biología, Universidad de Murcia, 30071 Murcia, Spain. Telephone: +34-868887148. Fax: +34-868883963. E-mail: vgarre@um.es.

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Table 1. Oleaginous Microorganisms Used for Single-Cell Oil Production

Microorganism		Oil content (% dry wt)	Reference
Microalgae	*Botrycoccus braunii*	25-75	7,8
	Chlorella vulgaris	40-60	9
	C. emersonii	63	9
	C. protothecoides	23	9
	C. sorokiniana	22	9
	Nannochloris sp.	20-35	8
	Nannochloropsis sp.	31-68	8
	Neochloris oleoabundans	35-54	8
	Nitzschia sp.	45-47	7,8
	Schizochytrium sp.	50-77	7,8
Bacteria	*Arthrobacter sp.*	>40	7
	Bacillus alcalophilus	18-24	7
	Gordonia sp.	93	10
	Rhodococcus opacus	96	10
Yeasts	*Candida curvata*	58	7
	Cryptococcus albidus	65	7
	Lipomyces starkeyi	64	7
	Rhodotula glutinis	72	7
Fungi	*Aspergillus oryzae*	57	7
	Cunninghamella echinulata	40-57	12,13
	Mortierella isabellina	60-86	7,11,13
	Mucor circinelloides	20	14

echinulata, which may accumulate up to 86 and 57% of lipids in dry biomass, respectively.[11−13] These fungi are able to grow and accumulate large amounts of lipids in cultures containing raw glycerol derived from biodiesel production as a carbon source. Glycerol is the major byproduct of the biodiesel production, and its recycling to produce oleaginous microbial biomass could significantly decrease the cost of biodiesel production.[13]

Biodiesel is conventionally produced by transesterification of extracted triacylglycerides with methanol, but a single-step method has been developed that transforms lipids present in dried microbial biomass into FAMEs, without previous lipid extraction.[16] This method combines the lipid extraction, the acid-catalyzed transesterification of the extracted saponifiable lipids, and the acid-catalyzed esterification of the extracted free fatty acids in one step and was initially proposed because of the substantial reduction in both time and solvents that this technique offers for analytical purposes.[17] Similar procedures that avoid the lipid extraction step have already been developed.[13,18−20] However, most of them involve a previous transmethylation step and do not include an acid-catalyzed transesterification and esterification.[13,18,19]

Biodiesel quality depends upon the fatty acid composition of raw materials, and consequently, not all microorganisms can be used as a feedstock for biodiesel production.[4,5] Thus, a careful characterization of the lipid composition of each microbial candidate should be carried out before its adoption by the industry. One way to generate microorganisms with ideal lipid composition for biodiesel production could be by means of genetic manipulation of key genes.[4,5] However, microorganisms considered thus far as a feedstock for biodiesel production lack appropriate genetic engineering techniques to improve fatty acid profiles that would produce high-quality biodiesel.[16] Besides, their genomes have not been sequenced, which makes it even more difficult to improve strategies based on genetic manipulation.

In contrast, the oleaginous fungus *Mucor circinelloides*, which was used for the first commercial production of microbial lipids,[21] has its genome sequenced and a large collection of genetic engineering techniques for its manipulation. These techniques include the expression of genes using autoreplicative plasmids and inactivation of genes by disruption[22] or gene silencing (RNAi).[23] In addition, the regulation of lipid accumulation in this fungus has been extensively studied for decades,[24,25] and key genes have been identified.[26] Moreover, the possibility to manipulate lipid accumulation in *M. circinelloides* using genetic engineering techniques has been recently proven. Thus, overexpression of malic enzyme, which has been postulated to be the rate-limiting step for fatty acid biosynthesis in *M. circinelloides*, led to a 2.5-fold increase in lipid accumulation.[27]

The *M. circinelloides* lipids extracted for mycelium grown in a solid medium have been suggested as a suitable feedstock to produce biodiesel.[14] Biodiesel was produced by acid-catalyzed transesterification/esterification because of its high free fatty acid content (31.6 ± 1.3%) following two different approaches: transformation of extracted microbial lipids and acid-catalyzed direct transformation of microbial dry mass. The FAME yield was significantly higher in the direct transformation than in the two-step process, with the FAME purity also being higher in the direct method. However, growth in a solid medium is unfeasible for the industry, which should use biomass from submerged cultures. Therefore, we describe here the characterization of the lipids accumulated by *M. circinelloides* mycelia grown in submerged liquid cultures and the acid-catalyzed direct transformation of the *M. circinelloides* biomass into biodiesel, without previous extraction of those lipids. In addition, we also show that the biodiesel obtained complies with the current existing standards, the ASTM D6751 standard in the United States and most of the specifications in the EN 14213 and 14214 standards in the European Union.

2. Experimental Section

2.1. Strains and Growth Conditions. The strain MU241,[28] derived from R7B[29] after replacement of its *leuA* mutant allele by a wild-type allele, was used as a wild-type strain to produce fungal biomass. For biomass production, 1×10^5 spores/mL

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were inoculated in a 500 mL flask with 100 mL of YNB2XG liquid medium (20 g/L glucose, 1.5 g/L ammonium sulfate, 1.5 g/L glutamic acid, 0.5 g/L yeast nitrogen base without amino acids and ammonium sulfate, 1 mg/L nicotinic acid, and 1 mg/L thiamine at pH 4.5) and incubated in the dark for 24, 48, 72, or 96 h at 26 °C and 250 rpm. Culture pH was measured every 24 h and manually adjusted by the addition of 1 M NaOH.

2.2. Analysis of Cell Lipids. Mycelia harvested by filtration using Whatman Paper No. 1 were dried between paper towels, frozen in liquid nitrogen, lyophilized, weighed to estimate dry mass, and ground using a mortar and pestle. Cell lipids were extracted as previously described.[30]

Characterization of cell lipids was performed following standard methods when possible. Free fatty acids, tri-, di-, and monoglycerides, FAMEs, carotenoids, sterol esters, sterols and tocoferols, retinoids and polar lipids in microbial oil were identified and quantified by TLC analysis. Chromatographic separation was developed in 20 × 20 cm silica-coated aluminum plates (Alugram Sil G/UV, Macherey-Nagel GmbH, Düren, Germany) using a solvent mixture of 88% (v) *n*-hexane, 11% (v/v) diethyl ether, and 1% (v/v) glacial acetic acid. Visualization was carried out by staining with iodine. Digital image analyses of staining plates were performed with Un-Scan-It Gel 6.1 software (Silk Scientific, Inc., Orem, UT), and the lipid compositions were quantified by the corresponding calibration curves.

Free fatty acid content in the lipid fraction extracted from the microorganisms was measured following a colorimetric procedure[31] based on the formation of cupric soaps and further quantification of the chromophore complex by absorbance at 715 nm in a Cary 500 spectrophotometer (Varian, Inc., Palo Alto, CA).

The phosphorus content in microbial oil was determined by inductively coupled plasma−optical emission spectrometry (ICP−OES) using a Vista AX model (Varian, Inc.). The analysis was performed according to EN 14107:2003 standard.

Fatty acid profiles of microbial, rapeseed, and sunflower oils were performed by gas chromatography (GC) in a CP-3800 gas chromatograph (Varian, Inc.) fitted with a flame ionization detector (FID) and TRB-FFAP capillary column (30 m length, 0.32 mm internal diameter, and 0.25 μm film thickness, Teknokroma, Barcelona, Spain). Prior to GC analysis, the oil samples were transformed into their corresponding methyl esters by saponification in 0.5 M KOH in methanol solution (30 min at 90 °C) followed by treatment with 14% boron trifluoride in methanol (10 min at 90 °C) and extraction with *n*-hexane/water. Finally, 3 μL of the organic phase containing FAMEs was injected into the capillary column, where the separation was achieved using a temperature ramp (1 °C/min) from 150 to 240 °C at a flow rate of 1 mL/min (injector temperature, 180 °C; detector temperature, 280 °C; injection mode, splitless). Identification of chromatographic peaks was performed by a comparison to a FAME standard mixture (reference 07131-1AM, Supelco, Bellefonte, PA) and quantification by means of external standards and their corresponding calibration curve. The iodine number was calculated as described in EN 14214:2003 standard from the free fatty acid profile.

2.3. Direct Acid-Catalyzed Transesterification/Esterification Reactions. *M. circinelloides* biomass was transesterified/esterified by stirring (900 rpm) with a solution of the catalyst (BF_3, H_2SO_4, or HCl) in a closed container at 65 °C for 8 h. In this direct process, a 10:1 methanol/chloroform (v/v) mixture was used as a reagent−solvent system, where the appropriate amount of the corresponding acid catalyst was dissolved. The obtained mixture was diluted with water and then extracted with hexane and diethyl ether using a centrifuge. The solvents were removed in a rotary evaporator, and the residue (FAMEs) was weighed to calculate the yield and then analyzed to determine its quality as biodiesel, following standard methods according to European Union specifications (EN 14214).

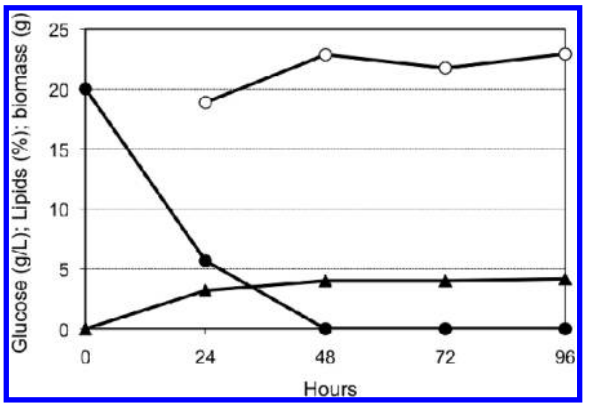

Figure 1. Kinetics of biomass production (▲), lipid biosynthesis (○), and glucose consumption (●) in *M. circinelloides* cultures. Data are presented as mean values from duplicate experiments.

3. Results and Discussion

3.1. Biomass Production and Lipid Characterization. To produce biodiesel, *M. circinelloides* biomass was obtained from the prototrophic strain MU241 grown in a liquid medium (YNB2XG) containing glucose as a carbon source (20 g/L). In our experimental conditions, the fungus grew very quickly because it consumed all of the available glucose and stopped growing in the first 48 h after inoculation (Figure 1). Similar fast growth has been observed in not only *M. circinelloides*,[26] but also other Mucorales, such as *M. isabellina*.[32] Lipid accumulation was high in the first analyzed time (24 h) and only increase slightly afterward. Although culture kinetic comparisons are difficult, particularly when different strains or culture conditions are used, similar lipid accumulation kinetics were previously observed in cultures of *M. circinelloides*.[26] In addition, the fatty acid profile of the lipid extracted from *M. circinelloides* did not change significantly with the fermentation time (data not shown).

After 96 h of growth, the fungus was clearly in stationary phase and no further increases in lipids were expected. In that time, a 4.17 ± 0.25 g/L fungal biomass with a total lipid content of 22.9 ± 0.9% dry mass was obtained. Nonetheless, not all lipids obtained from microbial biomass are suitable for making biodiesel. Only saponifiable lipids and free fatty acids (also referred to as oils) can be converted into FAMEs, which can be used as biodiesel if they comply with the current standards (ASTM D6751 in the United States or EN 14213 and 14214 in the European Union). The saponifiable lipids and free fatty acids (including energy storage and structural lipids) were 98.0 ± 1.3% of the total lipids extracted from *M. circinelloides* biomass, with the main components being triglycerides, polar lipids (phospholipids, sphingolipids, and saccharolipids), and free fatty acids (Table 2). In particular, the quantity of sphingolipids and saccharolipids produced by *M. circinelloides* was very high (around 54% of total lipids). The amount of neutral lipids (mono-, di-, and triglycerides) accumulated by *M. circinelloides* was 23.8%. Neutral lipids were comprised of mainly triglycerides (22.6 ± 1.3%). In addition, the proportion of phospholipids in this

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Table 2. Composition of the Lipids Extracted from *M. circinelloides* after 96 h of Growth

Lipid Classification	Type of Lipid	Concentration (%)
Free Fatty Acids and Saponifiable Lipids	Free Fatty Acids	3.6 ± 0.6
	Triglycerides	22.6 ± 1.3
	Diglycerides	0.8 ± 0.2
	Monoglycerides	0.42 ± 0.01
	Phospholipids	16 ± 1
	Sphingolipids and Saccharolipids	53.9 ± 1
	Sterol Esters	0.7 ± 0.2
	Total Saponifiable Lipids	98.04
Non-saponifiable Lipids	Carotenoids	1.30 ± 0.02
	Sterols and tocopherols	0.11 ± 0.01
	Retinoids	0.55 ± 0.08
	Total Non-saponiable Lipids	1.96

fungus was 16%. Significantly lower proportions of structural lipids (sphingolipids, saccharolipids, and phospholipids) were observed in the biomass from stationary cultures of other oleaginous fungi, such as *Cunninghamella echinulata*,[33] whereas the amount of neutral lipids (storage lipids) was higher at this stage. The level of neutral lipids (storage lipids) increased with time during the cultivation of this fungus, which means a decrease in the relative proportion of all of the structural lipids with this variable. In fact, the amount of structural lipids in a microorganism is concrete, and therefore, it has to keep constant with time. In contrast, lipid accumulation in *M. circinelloides* was 18.9% at 24 h, increasing only slightly after this time (Figure 1). In this case, the quantity of neutral lipids did not change significantly with the fermentation time, which justifies the relative high proportion of phospholipids, sphingolipids, and saccharolipids at the stationary stage. Although free fatty acid levels were still high (3.6 ± 0.6%), they were substantially reduced in comparison to those observed in biomass from solid medium (31.6 ± 1.3%).[14] The non-saponifiable lipid fraction, which consisted of small amounts of carotenoids, sterols, tocopherols, and retinoids (Table 2), was also reduced in these culture conditions (1.96%) in comparison to the solid medium (13.5%), probably because of the absence of light.[22] These results suggest that the fungal biomass from liquid cultures in the dark shows better characteristics for biodiesel production than that from solid cultures.

3.2. Biodiesel Production. The high concentration of free fatty acids (3.6 ± 0.6%) in *M. circinelloides* determines that an acid-catalyzed process is more suitable for producing biodiesel than an alkali one to avoid yield losses from free fatty acid neutralization.[34] Methods for simultaneous lipid extraction and transesterification involving a previous transmethylation step have been previously used with zygomycetes fungi, but they were avoided because of their low yields.[13] Therefore, the acid-catalyzed direct transformation method[16,17] (Figure 2) was applied to dried mycelial biomass using methanol and chloroform as solvents and H_2SO_4, HCl, and BF_3 as acid catalysts, all of which are commonly used in esterification or transesterification reactions.[35−38] Operating conditions (temperature, time, and solvent ratio) were previously optimized using *M. circinelloides* biomass from solid medium.[14] Using optimal reaction conditions (8 h at 65 °C), biodiesel yields were 18.9, 18.9, and 18.4% relative to the dry mass of *M. circinelloides*, using H_2SO_4, HCl, and BF_3, respectively. These yields were even slightly higher than the corresponding theoretical yield calculated for this microorganism (18.1%), indicating that acid-catalyzed direct transesterification/esterification of fungal biomass can be applied to *M. circinelloides* biomass from submerged cultures because it improves the amount of total lipids extracted in comparison to the conventional methods for lipid extraction from microorganisms.[30,39] This observation is supported by previous works describing increased recovery of fatty acids from microorganisms by direct transterification techniques.[17,40] Interestingly, these results also indicate that saponifiable lipids other than triglycerides, such as phospholipids, sphingolipids, and saccharolipids (Table 2), are transformed into FAMEs by this method and should be considered as substrates for FAME obtention.

At the end of the procedure, methanol and chloroform were recovered and recirculated through the process (Figure 2).

3.3. Quality Analysis of the Biodiesel. The quality of the biodiesel produced in the one-step procedure was determined according to the EN 14214 specifications, and the results were compared to the corresponding specified biodiesel limits in standards EN 14213 (European Union), EN 14214 (European Union), and ASTM D6751 (United States). Dependent upon the catalyst, the ester content ranged between 99.0 and 99.2% (Table 3), which is significantly higher than the corresponding specified minimum value in the European Union standard (96.5%). These values were higher and the reaction was faster than those reported for other oleaginous microorganisms, in which an acid-catalyzed direct transformation method was also used.[16] Futhermore, the amounts of all byproduct analyzed were below the maximum allowed values for American and European standards. Thus, the contents of individual glycerides (mono-, di-, and triglycerides) were within the biodiesel specifications, indicating that the transesterification and esterification reactions were complete. The free glycerol content was lower than the two standard limits, indicating that the glycerol residues were eliminated during the purification treatment. Besides, the individual glyceride and free glycerol levels were below the established limits. The total glycerol content also met all of the standards. The acid values, which depend upon the free fatty acid content, were also within the specifications in all reactions. In addition, non-saponifiable lipids were not detected in the *M. circinelloides*-derived biodiesel, which means that these types of lipids were also eliminated during the purification stage. Nonetheless, the biodiesel obtained had small quantities of polar lipids, which were lower than 0.9% in all cases (Table 3). These compounds are residuals of nonconverted polar lipids, and they are not considered in the biodiesel specifications established thus far.

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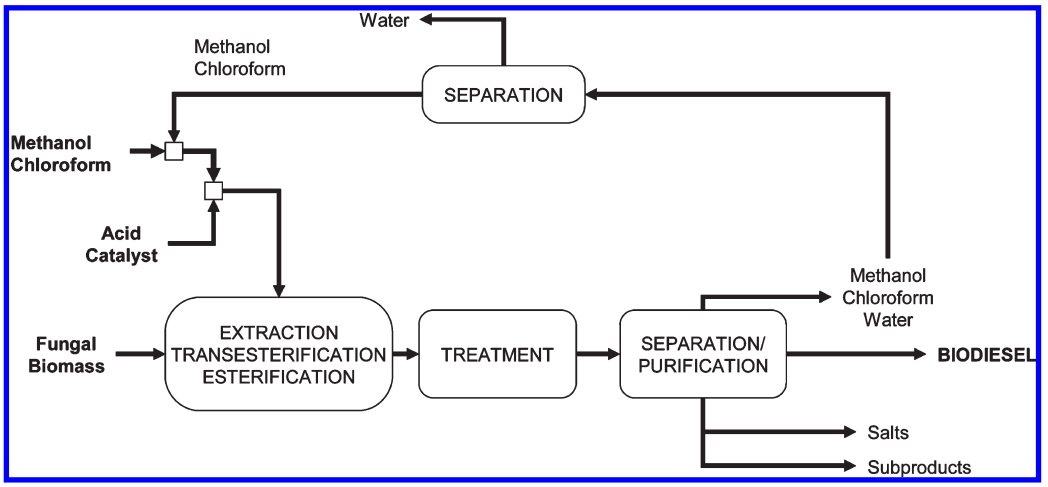

Figure 2. Schematic diagram of the process for biodiesel production from fungal biomass.

Table 3. Quality Control of *M. circinelloides*-Derived Biodiesel[a]

	catalyst				
property	BF_3	H_2SO_4	HCl	EU standard EN 14214	U.S. standard ASTM D6751
monoglyceride content (wt %)	nd	nd	nd	0.8 maximum	ns
diglyceride content (wt %)	nd	nd	nd	0.2 maximum	ns
triglyceride content (wt %)	nd	nd	nd	0.2 maximum	ns
free glycerol (wt %)	0.0020	0.0032	0.0030	0.02 maximum	0.02 maximum
total glycerol (wt %)	0.0020	0.0032	0.0030	0.25 maximum	0.24 maximum
acid value (mg of KOH/g)	nd	0.40	nd	0.5 maximum	0.5 maximum
non-saponifiable lipids (wt %)	nd	nd	nd	ns	ns
polar lipids (wt %)	0.8	0.8	0.9	ns	ns
ester content (wt %)	99.2	99.0	99.1	96.5 minimum	ns

[a] nd, not detected; ns, not a specified limit.

Table 4. Fatty Acid Composition in Biodiesel from *M. circinelloides*, Rapeseed, Sunflower, Palm, and Soy Oils

		content (wt %)				
fatty acid		*M. circinelloides* oil	rapeseed oil	sunflower oil	palm oil[41]	soy oil[41]
lauric acid	12:0	nd	nd	nd	0.1	nd
myristic acid	14:0	1.6	0.1	nd	0.7	nd
myristoleic acid	14:1	0.6	nd	nd	nd	nd
pentadecanoic acid	15:0	2.5	nd	nd	nd	nd
palmitic acid	16:0	20.7	5.0	6.3	36.7	11.3
palmitoleic acid	16:1	1.1	nd	0.2	0.1	0.1
stearic acid	18:0	7.0	1.6	2.2	6.6	3.6
oleic acid	18:1	28.0	36.3	20.6	46.1	24.9
linoleic acid	18:2	12.7	19.8	52.8	8.6	53.0
linolenic acid	18:3	22.5[a]	7.8[b]	3.5[b]	0.3[b]	6.1[b]
arachidic acid	20:0	0.3	0.1	1.6	0.4	0.3
gadoleic acid	20:1	nd	9.1	0.3	0.2	0.3
behenic acid	22:0	0.4	nd	7.2	0.1	nd
erucic acid	22:1	0.07	20.2	5.1	nd	0.3
lignoceric acid	24:0	1.2	nd	0.2	0.1	0.1
nervonic acid	24:1	nd	nd	nd	nd	nd
other		1.3	nd	nd	nd	nd
iodine value (g of I_2/100 g)		106.0	107.7	122.4	55.6	129.7

[a] The γ-linolenic acid isomer was obtained. [b] The α-linolenic acid isomer was obtained.

The fatty acid profile for the FAMEs obtained from *M. circinelloides* was compared to those produced for rapeseed, sunflower, palm,[41] and soy[41] oils (Table 4), which are the most commonly used raw materials by the biodiesel industry in Europe and the United States. Microbial oils usually differ from most vegetable oils in being quite rich in polyunsaturated fatty acids.[8] However, the content of these fatty acids in the biodiesel obtained from *M. circinelloides* was within the European Union specifications because the specified limit (1%) only includes polyunsaturated fatty acids with four or more double bonds, which are absent in *M. circinelloides*-derived biodiesel. FAMEs from *M. circinelloides* contained 12.7 and 22.5% of linoleic (two double bonds) and linolenic (three double bonds) acids, respectively, which would have low oxidative stability. In fact, the linolenic acid methyl ester content in the *M. circinelloides*-derived biodiesel was above the specified limit, 12%, in the European standards. On the

(41) Ramos, M. J.; Fernández, C. M.; Casas, A.; Rodríguez, L.; Pérez, A. *Bioresour. Technol.* **2008**, *100*, 261–268.

other hand, the high degree of unsaturation inherent to methyl esters from these fatty acids would evidence excellent fuel properties at low temperatures, which is an advantage in winter operation.[42] Moreover, all of these fatty acids are common in industrial vegetable oils, and in particular, sunflower and soy oils are also very rich in polyunsaturated fatty acids. Thus, the calculated iodine value, which is a measure of the total unsaturation level, for the *M. circinelloides*-derived biodiesel (106.0 mg of I_2/g) was far below the specified limit (120 mg of I_2/g) in the European Union standards and also met the United States standards because these specifications do not include the iodine value as a quality parameter. In comparison to the vegetable oils, the iodine value was very similar to the one obtained in biodiesel from rapeseed oil (107.7 mg of I_2/g), which is the preferred raw material for biodiesel production in Europe.

4. Conclusions

The results shown here indicate that *M. circinelloides* biomass from submerged cultures may be a suitable feedstock for biodiesel production. Moreover, the analyzed properties of the *M. circinelloides*-derived biodiesel fulfilled the specifications established by the current existing standards, ASTM D6751 in the United States and EN 14213 and 14214 in the European Union. In addition, efficient biodiesel production by direct transformation of fungal biomass without lipid extraction is technically feasible in *M. circinelloides*, which represents a starting point for developing this process on an industrial scale. However, biodiesel yields should be increased to make the industrial process economical, which could be attained by the genetic manipulation of this fungus. In this sense, efforts are now dedicated to overexpress genes that code for enzymes postulated to be rate-limiting steps for fatty acid biosynthesis in oleaginous fungi.[26] Other strategies are focused on the generation of strains with enhanced ability to use crop residues or industrial byproduct, avoiding competition with the food supply, with low linolenic acid levels or overexpressing genes involved in saponifiable lipid biosynthesis. Particularly interesting is the generation of strains with low free fatty acid levels because they could be used for biodiesel production by using a base-catalyzed technology, which is the common way to produce biodiesel on an industrial scale.

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(42) Vicente, G.; Martínez, M.; Aracil, J. *Bioresour. Technol.* **2004**, *92*, 297–305.