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Article

Single Cell Oil Producing Yeasts *Lipomyces starkeyi* and *Rhodosporidium toruloides*: Selection of Extraction Strategies and Biodiesel Property Prediction

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Abstract: Single cell oils (SCOs) are considered potential raw material for the production of biodiesel. *Rhodosporidium* sp. and *Lipomyces* sp. are good candidates for SCO production. Lipid extractability differs according to yeast species and literature on the most suitable method for each oleaginous yeast species is scarce. This work aimed to investigate the efficiency of the most cited strategies for extracting lipids from intact and pretreated cells of *Rhodosporidium toruloides* and *Lipomyces starkeyi*. Lipid extractions were conducted using hexane or combinations of chloroform and methanol. The Folch method resulted in the highest lipid yields for both yeasts (42% for *R. toruloides* and 48% for *L. starkeyi*). Also, this method eliminates the cell pretreatment step. The Bligh and Dyer method underestimated the lipid content in the tested strains (25% for *R. toruloides* and 34% for *L. starkeyi*). Lipid extractability increased after acid pretreatment for the Pedersen, hexane, and Bligh and Dyer methods. For *R. toruloides* unexpected fatty acid methyl esters

(FAME) composition were found for some lipid extraction strategies tested. Therefore, this work provides useful information for analytical and process development aiming at biodiesel production from the SCO of these two yeast species.

Keywords: single cell oil; lipid extraction; biodiesel; *Lipomyces starkeyi*; *Rhodosporidium toruloides*

1. Introduction

Environmental problems, including greenhouse gas emissions caused by the use of fossil fuels and the need for sustainable development, have turned attention to the utilization of renewable fuels [1]. In the transportation sector, which accounts for a significant part of the total consumption of energy, reaching more than 30% of the total energy consumption in the European Union (EU), liquid fuels are the most commonly utilized energy carriers [2]. Bioethanol and biodiesel are the two most important renewable fuels for replacing petroleum-based liquid fuels. Biodiesel consists of a mixture of fatty acid methyl esters (FAMEs), which are produced by the transesterification of triacylglycerols (TGAs) with alcohols in the presence of a catalyst [3,4]. TGAs utilized as raw material for biodiesel production can be plant oils (such as soybean and canola oil) and animal fats and represent up to 75% of the cost of biodiesel production [5]. However, the increased demand for these oils and fats for human food and animal feed and fuel production is causing rapid increases in their current prices [6].

This food-fuel competition and high raw lipid costs have led to a search for alternative TGA sources for biodiesel production. Single cell oils (SCOs) are potential candidates [7]. SCOs are lipids produced by oleaginous microorganisms including bacteria, yeast, fungi, and algae that are capable of accumulating at least 20% of their dry cell mass as lipids [8]. Their production does not compete with the food supply, since it can utilize agro-industrial wastes and by-products [9], such as lignocellulosic biomass [10]. Other advantages of using SCOs rather than plant oils are that arable land is not required, environmental parameters (such as weather conditions) are irrelevant, and cultivation times are shorter than those for the crops [4,11]. Although price of biodiesel from SCO is higher than first generation biodiesel, it is expected to decrease as the technology matures [12]. Furthermore, reduction of its cost can be achieved if zero or negative value waste substrates (as agricultural wastes) are employed as carbon and nitrogen sources [13] and if high valuable co-products produced concomitantly with SCO by the microorganisms are sold, such as carotenoids, polyunsaturated fatty acids and coco-butter equivalents [12,13].

Yeasts offer advantages over other oleaginous microorganisms, such as the availability of more candidates than with bacteria, oil compositions that are more suitable for biodiesel production than those obtained with bacteria and fungi, and shorter cultivation times and less complicated scale-up than those with autotrophic algae [4,14,15].

Despite the relatively large body of literature on the use of oleaginous yeasts for SCO production, there is no standard method of choice for neither its extraction aiming at lipid quantification [16,17] nor for process development. Most of the related literature is aimed at lipid extraction from algae with very few reports concerning oleaginous yeasts [16,18–20].

Most of the lipid extraction methods generally involve cell breakage and extraction with organic solvents. A large variety of solvents have been described for the extraction of SCOs; these include hexane alone [18,21] or mixed with isopropanol [21,22] and combinations of methanol and chloroform, as in the Pedersen [16] and the classical methods of Folch *et al.* [23] and Bligh and Dyer [24]. An efficient extraction requires the solvent to fully penetrate the cell mass and have a polarity similar to that of the target compounds [11]. Yeasts have some characteristics that can make lipid extraction difficult, such as their dense cell wall, which can be recalcitrant to many solvents, especially because neutral lipids are stored intracellularly in lipid bodies [15]. Also, lipid extractability differs according to the species, physical properties of the cell, and lipid composition of the SCO [19].

According to Pedersen [16] and Suzuki *et al.* [19] some sort of pretreatment has to be applied to the yeast cell in order to increase the ability of a solvent to extract the SCO. The purpose of pretreatment is to partially disrupt the structure of the cell wall to make the intracellular SCO more accessible to the solvent. However, very harsh pretreatment conditions can lead to SCO degradation. Different pretreatments have been evaluated separately or together, such as the addition of base [20], acid [19,25,26], and enzyme [18] and the application of sonication [27] and microwaves [18].

According to Espinosa-Gonzalez *et al.* [28] only a few yeast lipid extraction approaches have been scaled-up, and their application is restricted to the purification of value-added lipids or metabolites. Lipid extractions with hexane alone or combined with ethanol or isopropyl alcohol are preferable in most of the process development for biodiesel production from yeast oil [29–34].

Rhodosporidium toruloides, a red basidiomycete, and *Lipomyces starkeyi*, an ascomycete, are both considered to be excellent candidates for the production of SCO as they can accumulate up to 70% of their dry cell mass as SCO and grow on a wide range of raw materials [15], including lignocellullosic hydrolysates [35,36]. However, *L. starkeyi* and *R. toruloides* differ not only morphologically, but also in cell wall composition and structure. The first one is a round-shaped cell and its wall is mainly composed of mannose, galactose, and glucuronic acid. *R. toruloides* has an elongated shape and a multilayer cell wall made up of glucose, mannose, galactose, and glucosamine [37]. Dissimilarities in cell shape and structure can cause differences in effectiveness of oil recovery [11].

The present work aims to evaluate SCO extraction strategies from intact and chemically or enzymatically pretreated cells of *L. starkeyi* and *R. toruloides*. We evaluated the most cited methods in the literature—the Folch, Pedersen, and Bligh and Dyer methodologies—and a hexane-based method—the preferable solvent for larger scale SCO extractions. The results obtained indicate the importance of evaluating the most suitable approach for lipid extraction for each yeast species, as the lipid yield varied considerably according to the solvent and pretreatment used. Although hexane is the preferable solvent for large scale application, the results suggested that cell pretreatment or mechanical disruption step are necessary when using this solvent.

2. Results and Discussion

2.1. Yeast Cultivation for Lipid Production

The batch cultivation of *Rhodosporidium toruloides* and *Lipomyces starkeyi* resulted in 13.3 g/L and 21.2 g/L of final cell mass and maximum specific growth rates (μ_{max}) of 0.09 and 0.08 h^{-1},

respectively. These values can be considered to be in accordance with values already described in the literature for the use of xylose or a combination of xylose and glucose as carbon source (10–17 g/L as final cell mass and μ_{max} ranging from 0.04 to 0.14 h^{-1}) [34,38]. Both yeasts also consumed xylose and glucose simultaneously. Yeasts with this ability can reduce the overall costs of lipid production by using the cellulose and hemicellulose components of cell mass hydrolysates as a substrate.

2.2. Lipid Extraction from Intact and Pretreated R. toruloides and L. starkeyi Cells

The Folch method with intact cells gave the highest lipid yield for both species (42% *w*/*w* and 47% *w*/*w* lipid yield for *R. toruloides* and *L. starkeyi*, respectively) (Table 1). This combination of chloroform and methanol (2:1) seems to successfully penetrate the cell wall and disrupt the strong hydrogen bonds between membrane lipids and proteins [39]. Therefore, cell pretreatment, a time-consuming step [17], seems to be unnecessary for lipid extraction with the strains used in this work. Also, according to Yu *et al.* [25] pretreatment with acids, alkali, and enzymes has limited application on a larger scale due to its relatively high cost and equipment corrosion. Although the Folch method is very convenient for lipid analytical quantification, chloroform and methanol are described as prohibitive for large scale lipid extractions, due to their toxicity, higher cost (when compared to hexane) and for being environmentally unfriendly [28].

Table 1. Effect of different pretreatments and extraction methods on lipid yield of *R. toruloides* and *L. starkeyi*.

Solvent	PT	*R. toruloides*	*L. starkeyi*
		Lipid Yield (% *w*/*w*)	Lipid Yield (% *w*/*w*)
C:M (2:1) Folch *et al.* [23]	acid	34 ± 15 *	48 ± 3 **
	enz	31 ± 2 **	37 ± 0 **
	none	42 ± 1 **	47 ± 1 **
C:M (1:1) Pedersen [16]	acid	34 ± 7 *	52 ± 7 ***
	enz	27 ± 7 ***	32 ± 0 **
	none	28 ± 2 **	38 ± 0 **
Hexane	acid	42 ± 2 **	40 ± 0 **
	enz	3 ± 0 **	3 ± 0 **
	none	3 ± 0 **	5 ± 1 **
C:M:W (2:2:1.8) Bligh and Dyer [24]	acid	25 ± 0 **	34 ± 7 ***
	none	23 ± 2 **	7 ± 1 **

Notes: Experiments carried out in: * quadruplicate, ** duplicate, or in *** triplicate; C: chloroform; M: methanol; W: water; PT: pretreatment; enz: enzymatic. Errors are expressed as average deviation.

Pedersen's method for *L. starkeyi* resulted in good extraction levels for acid-treated cells (although with a large variation) including the highest yield in this study (52% ± 10% *w*/*w*). Suzuki *et al.* [19] concluded that Pedersen's was the most suitable method for lipid extraction from freeze-dried *L. starkeyi* cells, resulting in 2.4 times higher lipid yields than those obtained with Folch's method. Pedersen's method for lipid extraction from *R. toruloides* resulted in intermediate yields despite the use of pretreatments (28%–34% *w*/*w*). No similar reports were found in the literature for comparison.

The extraction of acid-treated cells with hexane showed lipid yields (42% *w*/*w* and 40% *w*/*w* for *R. toruloides* and *L. starkeyi*, respectively) similar to those obtained using the Folch method. Extraction with intact and enzymatically treated cells resulted in very low lipid yields (below 6% *w*/*w* for both species). Jin *et al.* [18] also reported low lipid extraction yields for *R. toruloides* with hexane. Sheng *et al.* [39] attributed the low efficiency of hexane to its polarity being lower than that of chloroform and to its inability to damage the cell wall. Therefore, for the use of hexane for analytical or larger scale lipid extraction is necessary the use a cell pretreatment step or a mechanical method of disruption [14].

Extraction with the Bligh and Dyer [24] method, applied to intact and enzymatically treated cells, was severely hindered by the formation of stable emulsions making it impossible to extract lipids from enzyme-treated cells. The formation and stabilization of emulsions was probably caused by cellular constituents, including proteins, phospholipids, and glycolipids [18]. For intact cells, lipid extraction was only possible after reducing to one third the mass of dried cells, but lipid yields were still low (23% and 7% *w*/*w* for *R. toruloides* and *L. starkeyi*, respectively). The Bligh and Dyer method produced a reasonable lipid yield (39% *w*/*w*) only with acid-treated *L. starkeyi* cells.

There was a relative large mass loss for *R. toruloides* in the case of both pretreatments (40%–57%) and for *L. starkeyi* treated with acid (34%–41%). These mass losses were due in part to the action of the acid and the enzymes since they were expected to partially break the cell wall, which comprises 25%–30% of the dry cell mass of the yeast [17]. Loss of intracellular material and losses due to recovery of cell mass from the filters should also be taken into account. Enzymatic treatment of *L. starkeyi* (the case of the smallest mass losses, 6%–16%) was less efficient, most likely due to the sulfide bonds in its cell wall, which provide more stability and rigidity to this organelle [17]. This hypothesis is supported by the information gathered by Barnett and colleagues [37] on the need to add a thiol agent for the lysis of *L. starkeyi* with glucanases.

In general lipid yield on both yeast was around 40% to 50% (*w*/*w*) which implies in 60%–50% lipid-free cell mass. Aiming at process development, the lipid-free cell can possibly be further used as nitrogen source for new lipid production batches or sold as animal feed, thus improving the economics of biodiesel from microbial oils [14,40].

2.3. FAME Composition and Estimated Properties of Biodiesel from the Oil Extracted from R. toruloides and L. starkeyi

FAME composition of lipids obtained with different extraction methods and pretreatments are reported in Figure 1, along with average values from the review by Li *et al.* [4]. The predominant FAMEs generated from the SCOs extracted from both yeasts in this work were palmitic (C16:0) and oleic (C18:1) acid derivatives (over 70% of detected FAMEs). Palmitic, stearic (C18:0), linoleic (C18:2), linolenic (C18:3), and oleic acids are the most suitable for biodiesel production [41], especially oleic acid [42].

Cetane number (CN) is a diesel fuel quality parameter required for good engine performance, and the longer the fatty acid carbon chains and the more saturated the molecule, the higher the cetane number [43]. The minimum acceptable CN in the EU is 51 [44]. Cold filter plugging point (CFPP) is an estimation of the lowest temperature prior to the formation of waxy solids that settle and plug filters and fuel

lines. Acceptable values vary according to country climate and season [43]. FAMEs produced with lipids extracted from *L. starkeyi* were similar in all extraction strategies and to the average values from literature [4] (Figure 1a). The average composition was 40.0% of C16:0, 4.0% of C16:1, 5.0% of C18:0, 45.0% of C18:1, and 2.0% of C18:2. The estimated average CN of this oil was 60.2, a value in agreement with the required standards in the EU (CN ≥ 51) and the CFFP was 5.4 °C.

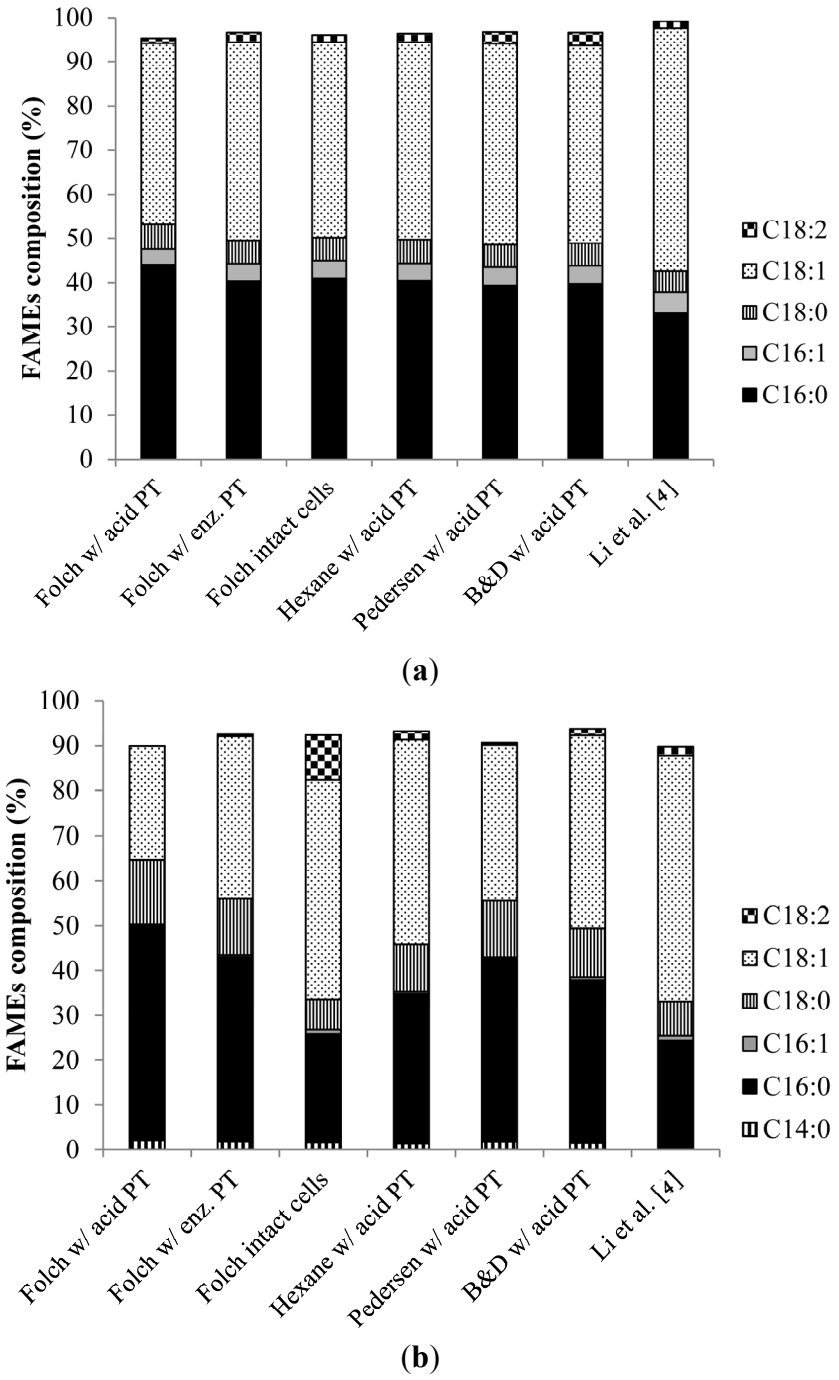

Figure 1. Comparison of the FAME compositions of lipids obtained with different extraction methods and pretreatments and values from the literature for (**a**) *R. toruloides* and (**b**) *L. starkeyi*. Only FAME contents above 1% were considered. PT: pretreatment; B&D: Bligh and Dyer; enz: enzymatic.

The composition of FAMEs produced with lipids from *R. toruloides* varied considerably according the lipid extraction strategies (Figure 1b). The lipid extraction strategies that resulted in the highest lipid yields for *R. toruloides* Folch with intact cells and hexane with acid-pretreated cells resulted also in FAME profiles (average values: 1.6% of C14:0, 28.6% of C16:0, 0.8% of C16:1, 8.6% of C18:0, 47.1% of C18:1, and 5.8% of C18:2) similar to the average composition reported by Li *et al.* [4] (24.3% of C16:0, 1.1% of C16:1, 7.7% of C18:0, 54.6% of C18:1, and 2.1% of C18:2). The estimated CN and CFPP values were 54.9 and 1.6 and 58.3 and 10.5 °C for the case of Folch and hexane strategies, respectively.

No explanation was found for the differences in FAME composition of the oil extracted with other methods in the literature for yeasts. Li *et al.* [45] reported a similar fact for the algae *Tetraselmis* sp., as different strategies for lipid extraction also resulted in different yields and different FAME profile. We can speculate that those methods (Folch, Pedersen, and Bligh and Dyer) applied to pretreated cells produced a selective oil extraction or the pretreatment was harsh enough to cause its degradation.

3. Experimental Section

3.1. Microorganisms, Media, and Chemicals

Rhodosporidium toruloides CCT 0783 was obtained from Coleção de Culturas Tropicais (Fundação André Tosello, Campinas, Brazil) and *Lipomyces starkeyi* CBS 1807, from Centraalbureau voor Schimmelcultures Fungal Biodiversity Centre (Institute of the Royal Netherlands Academy of Arts and Sciences, Utrecht, The Netherlands). Pre-inoculum and inoculum were prepared with yeast extract peptone dextrose (YPD) medium (20 g/L glucose, 20 g/L peptone, and 10 g/L yeast extract) at 200 rpm and 25 °C and 28 °C for *L. starkeyi* and *R. toruloides*, respectively. The medium for lipid production (pH 5.5) consisted of (g/L) glucose, 5; xylose, 65; $MgSO_4 \cdot 7H_2O$, 1.5; KH_2PO_4, 1.5; yeast extract (containing 4.4% amino nitrogen and 9.3% total nitrogen), 2; (NH4)2SO4, 0.5; and trace element solution, 1.0. Total C/N molar ratio was 116. Trace element solution was prepared according to Meesters *et al.* [46]. Media were sterilized at 121 °C for 15 min. Commercial enzyme complex from *Trichoderma harzianium* (Sigma Aldrich, St. Louis, MO, USA) solution was used for enzymatic pretreatment of cells at the concentration of 0.1 g enzyme/g dry cell mass (activities of 1070, 120, and 16 U/g solid cellulase, chitinase, and protease, respectively) in 0.1 mol/L phosphate buffer, pH 5.8. All chemicals and reagents were of analytical grade.

3.2. Yeast Cultivation for Lipid Production

Yeast cultivation started with pre-inoculum preparation: the activation of 1 mL of the stock of cells (kept in glycerol at −80 °C) in 9 mL YPD medium at 25 °C for 24 h. Inoculum was prepared by cultivation of the pre-inoculum in 250 mL of YPD medium for 48 h at 25 °C. Lipid production took place in a 3.0 L bioreactor (Applikon Biotechnology, Schiedam, The Netherlands) with a working volume of 2.5 L, an inoculum size of 10% (*v*/*v*), a stirring speed of 500 rpm, and an aeration rate of 1 volume of air per volume of liquid per minute (vvm). *R. toruloides* was cultivated at 28 °C and *L. starkeyi* at 25 °C. pH was monitored and kept at 5.5 by the addition of either 5 mol/L NaOH or 5 mol/L H_2SO_4. After 95 h the cultivation was stopped and the broth was separated into 50 mL aliquots. The cell mass of these aliquots was harvested by centrifugation (5804R, Eppendorf, Hamburg,

Germany) at 8228 *g* for 15 min, washed twice with distilled water and dried at 100 °C to constant mass. The dried cell mass of each aliquot was weighed and used in pretreatments or lipid extraction.

3.3. Pretreatments

Acid pretreatment of the yeast cells was carried out by the addition of 2 mol/L of HCl at a ratio of 12.5 mL per gram of dried mass and incubation at 80 °C for 1 h [35]. The cell mass was recovered and exhaustively washed with water using filter paper (120H, Munktell, Bärenstein, Germany) and the flow was filtered again using a 0.45 μm pore polytetrafluoroethylene (PTFE) membrane (Millipore, Bedford, MA, USA) in order to minimize the mass loss. The combined retained cell mass was used for extraction tests. Enzymatic pretreatment was performed with few modifications to the protocol described by Varavallo *et al.* [47] for protoplast preparation. Fifty milliliters of fresh enzymatic solution was added to the cell mass in the 50 mL aliquots (described in Section 2.2) and this cell suspension was incubated at 30 °C and 200 rpm for 3 h, followed by enzyme inactivation at 80 °C for 30 min. Cell mass was recovered and washed using filter paper (1001, Munktell, Bärenstein, Germany). In order to avoid the effect of water on solvent extraction and to quantify the mass loss due to the pretreatments and filtrations steps, the pretreated cell mass was dried again at 100 °C to constant mass.

3.4. Lipid Extraction

Four extraction methods (based on hexane and different combinations of chloroform and methanol) were evaluated for the extraction of lipids from intact and pretreated cells of *R. toruloides* and *L. starkeyi.* Extractions were done with 0.5–0.7 and 0.9–1.2 g of dried cell mass of *R. toruloides* and *L. starkeyi*, respectively, except when using the Bligh and Dyer method for intact cells of *R. toruloides* (0.2 g) and *L. starkeyi* (0.4 g). Excess solvent was used to avoid the need for re-extraction steps [48]. Briefly, 80 mL of hexane or chloroform-methanol at ratios of 2:1 [23] and 1:1 [16] were added to dried cells and incubated at room temperature. After 24 h, 16 mL of 9 g/L of NaCl solution were added to produce a liquid biphasic system consisting of nonlipidic material (light phase) and lipids solubilized in the extracting solvent (heavy phase) with cell debris at the liquid–liquid interface. The cell debris was removed by filtration with a 0.45 μm hydrophobic membrane (Millipore). The heavy phase was recovered and subjected to lipid quantification and FAME analysis. We used the classical protocol for the method known as Bligh and Dyer [24], since there are many modifications of the original method in the literature. This protocol consisted in resuspending the dried cell mass (intact and acid-pretreated dried cells) in 8 mL of water and adding a chloroform and methanol mixture at a volume ratio of 0.8:1:2 and mixing with a vortex for 2 min. Then 10 mL of chloroform was added and the mixture was agitated for 30 s. A biphasic system was formed after the addition of water to achieve a final proportion of 1.8:2:2 and mixing was continued for another 30 s. The liquid phase was separated from the cell mass by filtration with filter paper (#1, Whatman, Maidstone, UK). Thirty milliliters of chloroform were used to wash the cell mass and the filtrate was left at room temperature for phase separation. The chloroform layer (heavy phase) was recovered and extraction efficiency was assessed by lipid yield (mass of lipids/cell dry mass) and FAME composition. Experiments were carried out in duplicate except when average deviation exceeded 10%. In these cases triplicates or quadruplicates were used.

3.5. Analytical Methods

Cell mass growth was monitored by optical density at 600 nm and determined gravimetrically by drying at 100 °C for 24 h. Glucose and xylose were quantified using an HPLC system equipped with a refractive index detector (Perkin-Elmer, Foster City, CA, USA) and an Aminex HPX-87H column (BioRad, Hercules, CA, USA). The mobile phase was 0.005 mol/L H_2SO_4 at a flow rate of 0.6 mL/min, an injection volume of 20 μL, and a column temperature of 65 °C. Lipid content was measured gravimetrically after extraction and solvent evaporation under vacuum in a rotary evaporator (Buchi, Flawil, Switzerland) at 65 °C. The extracted lipids were methylated with boron trifluoride for fatty acid compositional analysis [49]. The methylated fatty acids were analyzed in a gas chromatograph system (Varian CP-3800, Stockholm, Sweden) with an autoinjector (CP-8410 AutoInjector, Varian, coupled to a capillary column (wall coated open tubular (WCOT) fused silica 100 m × 0.25 mm coating CPSIL 88 for FAME). Helium was used as a carrier gas at a flow rate of 30 mL/min. The injector and detector temperatures were 270 and 300 °C, respectively. The initial oven temperature was 175 °C for 26 min. It was then increased to 205 °C at a rate of 2 °C/min and held at 205 °C for 24 min. Fatty acid peaks were identified according to a standard mixture (GLC standard 461, Nu-Chek Prep, Elysian, MN, USA) and retention times. The Galaxie Chromatography Data System (Varian) was used for peak area integration using methyl 15-methylheptadecanoate as internal standard (Larodan Fine Chemicals AB, Malmö, Sweden).

3.6. Prediction of Biodiesel Properties

Cetane number (CN) was predicted according to Krisnangkura [50] as the weighed percentage of each methyl ester and its individual cetane number (CN_{ME}) using Equation (1):

$$CN_{ME} = 58.1 + 2.8 \left[\left(\frac{n_c - 8}{2}\right)\right] \times nD \quad (1)$$

where n_c is the number of carbon atoms and *nDB* is the number of double bonds of the fatty acid. The cold filter plugging point (CFPP) was calculated as a function of the long chain saturated factor (LCSF), as described in Ramos *et al.* [43], with Equations (2) and (3):

$$LCSF = 0.1 \times C16(wt\%) + 0.5 \times C18(wt\%) + 1 \times C20(wt\%) + 1.5 \times C22(wt\%) + 2 \times C24(wt\%) \quad (2)$$

where wt% corresponds to the amount of the specified methyl ester (in percentage):

$$CFPP = 3.1417 \times LCSF - 16.477 \quad (3)$$

4. Conclusions

The major conclusion of this work is that out the four SCO extraction methods tested, the method proposed by Folch *et al.* [23] was the best, not only in terms of lipid yield, but also in terms of FAME composition (composition closer to the expected for both yeasts studied based on literature). The Bligh and Dyer [24] method, widely cited in the literature related to yeast SCO, underestimated the lipid content in the strains tested in this work. We also showed that acid pretreatment increased lipid yields for the Pedersen [16] and hexane methods. Pretreatment or mechanical disruption seemed to be mandatory for any extraction using hexane.

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Author Contributions

All authors contributed jointly to all aspects of the work reported in the manuscript. All authors have read and approved the final manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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