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Keywords

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- Hydrolysis
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- Distillery waste water

Abstract

The present work deals with three methods proposed for the direct transesterification of lipids in the filamentous fungi *Aspergillus niger* and in the oleaginous yeast *Yarrowia lipolytica*, grown on distillery spent wash (DSW). For comparison, the reported amount of fatty acid methyl esters (FAMEs) generated by the improved methods applied to dry and wet biomass was normalized against the amount of FAMEs yielded via a two-step extraction/methanolysis method from the same samples. To increase the esterification reactions yield from wet biomass, modifications of the process were evaluated, including: the use of a co-solvent; hydrolysis pretreatment; and the use of a double catalyst. The methylated derivatives generated by each method were then analyzed by gas chromatography with flame ionization detector (GC-FID). It is shown that direct transesterification yielded higher amounts of FAMEs than the two-step methods in both microorganisms, with normalized FAMEs productions of: 146 % (*A. niger*) and 190 % (*Y. lipolytica*) from dry biomass, and 119 % (*A. niger*) and 143 % (*Y. lipolytica*) from wet biomass, respectively. In the case of wet *Y. lipolytica*, the double catalyst improvement approach generated 20 % higher FAMEs production than the direct acid methanolysis of the same material. The contributions of the methods parameters for improved esterification and for the FAMEs production profile for *Y. lipolytica* are also discussed.

ABBREVIATIONS

ASE: Accelerated Solvent Extraction; ALnAME: A-Linolenic Acid Methyl Ester; DSW: Distillery Spent Wash; FAME: Fatty Acid Methyl Ester; LAME: Linoleic Acid Methyl Ester; MUFA: Monounsaturated Fatty Acids; OAME: Oleic Acid Methyl Ester; PAME: Palmitic Acid Methyl Ester; Poame: Palmitoleic Acid Methyl Ester; PUFA: Polyunsaturated Fatty Acids; SFA: Saturated Fatty Acids; SCO: Single Cell Oil SAME: Stearic Acid Methyl Ester; UAM: Ultrasound Assisted Maceration

INTRODUCTION

Single cell oils (SCO), lipids of microorganismal origin, have gained significant attention, especially microalgal lipids that can be used to produce biofuels [1-3]. These feedstocks have inherent advantages over the edible crops that are currently used, such as their higher productivity thanks to their shorter life cycles, their ability to grow on industrial wastes [4] and their limited requirement for arable land. However, lipid production from these feedstocks suffers from specific drawbacks inherent to their growth suspended in water medium. The concentration of the microorganisms ranges from 1 g/l [5] to over 150 g/l [6] depending on the culture process and the species considered, with the lowest concentration for microalgae in open raceways and the highest concentrations for yeast biomass in fed- batch cultures. The high media water content requires dewatering steps to facilitate solvent extraction of lipids from the biomass, consequently increasing costs while decreasing the energy efficiency of the whole process [7]. Solutions have been proposed to enhance the efficiency of biodiesel production from microorganisms, focusing on eliminating the extraction step [8] and working directly on the wet biomass obtained after a first, low energy requiring, concentration step [7-10]. This improvement

effort is mostly focused on microalgal biomass because of its ability to upgrade inorganic carbon into valuable lipids under adequate light irradiance. However, biomass production under these conditions is relatively low compared with fungal and yeast biomass [5,6]. Methodologies developed for microalgae could also be useful for the development of biodiesel from yeast or fungal biomass. SCO for fuel production, particularly fatty acid methyl esters (FAME), are not yet economically feasible, partly due to the use of refined sources of nutrients for biomass production. Different wastes are currently being studied as more economical replacements for these costly nutrients, such as crude glycerol, palm oil mill effluent or serum latex [11]. Distillery spent wash (DSW), also known as vinasse is the liquid waste from alcohol distillation. It contains high levels of nutrients, as illustrated by its high chemical oxygen demand and its high total nitrogen content. These properties cause severe polluting effects when it is discharged into the environment [12]. As such, DSW could be used as a source of cheap nutrients for microbial growth, reducing the polluting capacity of the waste at the same time. *Aspergillus niger* grown on distillery stillage without supplementation has been shown to produce up to 25 g/l biomass (unpublished results). *Yarrowia lipolytica* is known oleaginous yeast, producing over 20 % dry weight of total lipids in its biomass in high C/N ratio culture medium, but only 4 g/l biomass on vinasse without supplementation. Transesterification of lipids from biomass is commonly carried out using a conventional two-step process, involving extraction of the lipids from the biomass followed by their catalytic trans-methylation. Using wet biomass directly after culture for transesterification process would eliminate the costly drying step and simplify the downstream processing in biodiesel production. Several attempts have been made using microalgae, based on pre- treatment of the biomass or modification of the methanolysis procedure [13-15], but very little work has been done on other microbial sources such as *Cryptococcus curvatus* [16] and, to our knowledge, none has been reported on biomass grown on sugarcane distillery wastewater. Here we worked on three modifications of the methanolysis process, namely co-solvent addition, pre-hydrolysis of the biomass and double catalyst methanolysis, which have been used in the improvement of FAME production from microalgae [13-15]. The filamentous fungus *Aspergillus niger* and the yeast *Yarrowia lipolytica* were chosen as model microorganisms to evaluate the applicability of the three biodiesel production processes using biomass grown on sugarcane vinasses. Firstly, different extraction methods were studied in order to choose a reference two-step transesterification method, namely Soxhlet extraction; ultrasound assisted maceration and accelerated solvent extraction. Then, the efficiency of the three biodiesel production methods was evaluated by comparison with the chosen reference two-step ultrasonic extraction/transesterification method on dry biomass and the improvements were assessed by comparison with the direct transesterification of wet biomass.

EXPERIMENTAL

Materials

Cultures of microorganisms: Cultures of *Aspergillus niger* (MUCL 19001) and *Yarrowia lipolytica* (MUCL 30108) were obtained from Mycothèque de l'Université Catholique de Louvain.

Standards and reagents: Standards for palmitic, palmitoleic, stearic, oleic, linoleic and α-linolenic acid methyl esters were products of Sigma-Aldrich (USA). Heptadecanoic acid methyl ester of chromatographic purity (Sigma-Aldrich, USA) was used as internal standard for chromatographic analyses. Other chemicals, all of analytical grade were: chloroform, methanol, heptane and NaOH from CarloErba (France) and sulfuric acid (95%) from Labosi (Fisher Scientific, USA).

Equipment: *Y. lipolytica* was grown in a 2 l Sartorius Biostat B+ bioreactor. Biomass was harvested using the centrifuge Heraeus Biofuge PrimoR from Thermo Scientific, with a Fixed Angle High conic rotor. Ultrasonication was performed on a Transsonic TI-H-10 ultrasonic bath. When needed, the samples were freeze dried with a Labconco Free Zone 2.5 for later analysis. The analytical instrument used was a CP3800 Gas chromatograph (Varian) with a flame ionization (FID) detector. The analytes were separated on a SG BPX-70 capillary column (50 m x 0.22 mm i.d. x 0.25 µm film thickness) using an oven temperature programmed to rise from 120°C to 230°C at 3°C/min.

Methods

Biomass production: The strains were grown at 28°C on sugarcane distillery spent wash without any additional nutrients for ten days in shaking flask culture for *A. niger* and in a 2 l bioreactor for *Y. lipolytica*. After cultivation, the strains were harvested by centrifugation at 7500 rpm (7800 g), 15 min, and 20°C then washed three times with distilled water. Half of the centrifuged biomass was stored at -80°C until needed while the other half was freeze-dried for later analysis.

Determination of the biomass lipid content: Transesterification of lipids from biomass is commonly carried out using a two-step process, involving extraction of the lipids from the biomass followed by their catalytic transmethylation. Different extraction methods were studied in order to select a reference two-step transesterification method, namely Soxhlet extraction, ultrasound assisted maceration (UAM) and accelerated solvent extraction (ASE). Freeze-dried biomass from the same cultivation batch was crushed using a mortar and pestle and used for comparison of the extraction methods using a mixture of chloroform: methanol (2:1, v/v) as the solvent. The operational conditions of these preliminary experiments are shown in Supplementary data. The ultrasound assisted maceration was established to be best extraction method (see additional support material for the selection procedure of the two-step esterification approaches for each microorganism). Briefly, freeze-dried biomass (100 mg) was mixed with 10 ml extraction solvent and sonicated at 45 kHz, 40°C and 80 % power for 15 min. Chloroform: methanol (2:1 v/v) mixture (3 ml) and distilled water (3 ml) was added for phase separation. The lipid extract and residue were separated by centrifugation at 2500 rpm (866 g) for 10 min. Two further cycles of solvent extraction were performed. The collected organic phases were dried on anhydrous Na_2SO_4 and solvent were removed under reduce pressure at 40°C. For each method, the total lipid content of the biomass was gravimetrically quantified as percentage of the dry weight.

Transesterification processes

Conventional two step dry extraction/transesterification: Figure (1) shows the six different transesterification methods assessed in this study. FAMEs from freeze-dried biomass obtained by the two-step ultrasound-assisted extraction was used as a reference for FAMEs content. The efficiency of other methods was expressed as a percentage of this value. Transesterification was performed according to a procedure described by Liu et al., [17]. Lipids from the ultrasound extraction method were transferred to round-bottom flasks using chloroform. The chloroform was evaporated and 5 ml of 2 % H_2SO_4-methanol (v/v) was added. The mixture was reflux heated at 90°C for 1 h under constant stirring. The flasks were then cooled to room temperature.

Direct transesterification processes

Direct Transesterification on Dry biomass: DTD: in order to define the best reaction time for the analysis, the time course of a transesterification reaction was monitored with 1 g dry biomass suspended in 50 ml methanol – 2 % H_2SO_4 (v/v) and reflux at 90°C for 2 hours under constant stirring (Figure 1). At regular intervals, 2 ml of samples was taken.

Direct Transesterification on Wet biomass

The moisture content of the biomass was assessed after the centrifugal washing steps by drying wet biomass at 100°C until no mass variation was observed. *Y. lipolytica* and *A. niger* moisture content was 63 % and 73 % respectively, of the wet biomass. The moisture content was used to obtain the weight of the wet material containing 1 g of dry biomass. The wet biomass was then processed as for dry biomass in one-step direct transesterification (Figure 1).

One-step methods for improved direct transesterification of wet biomass

Use of co-solvent: The co-solvent method was applied according to a previously described protocol [15]. Thirty millilitres of chloroform: methanol (2:1, v/v) was mixed with H_2SO_4 (2 % v/v). Wet biomass (100 mg dry basis) was added to the solvent preparation and heated at 90 °C for 1 hour under constant stirring. Distilled water was then added to the reaction solvent and the lower organic layer was separated by centrifugation, and vacuum evaporated to recover the FAMEs.

Hydrolysis pre-treatment: Hydrolysis pre-treatment was performed following a previously described method [14]. Wet biomass (100 mg dry basis) was subjected to hydrolysis with 300 ml H_2SO_4 (95 %) per kilogram of dry biomass for 30 minutes at 140°C. After cooling, 10 ml of methanol was added for methanolysis of the extracted lipids for 1 hour at 90°C under constant agitation.

One-pot sequential double catalyst method: The double catalyst method was performed according to a previously described protocol [13]. The first step involved basic catalysis on wet biomass (100 mg dry basis) with 5 ml NaOH (0.5 N) in methanol for 30 min at 90°C, followed by acid catalysis with 5 ml H_2SO_4 in methanol (5 % v/v) for another 30 min at 90°C under constant stirring.

Analytical methods

GC-FID analysis: Gas chromatography was used for the identification and quantification of the FAME present in the samples prepared using each tested methodology. 1 ml Heptane was mixed with the samples after FAME production, followed by distilled water. The upper heptanic layer was pipetted into dry Na_2SO_4 for dewatering, filtrated on a 0.2 µm filter and 20 µl of C17:0 methyl ester solution (2.75 mg/ml) was spiked into 200 µl heptanic mixture. 0.5 µl of spiked samples were analyzed by gas chromatography with an injector temperature of 240°C, a detector temperature of 260°C, a nitrogen flow rate of 1.3 ml/min, a split ratio of 5:1 at 0.5 min from the injection, then 50:1 at 5 min after injection. FAMEs were identified by comparing their

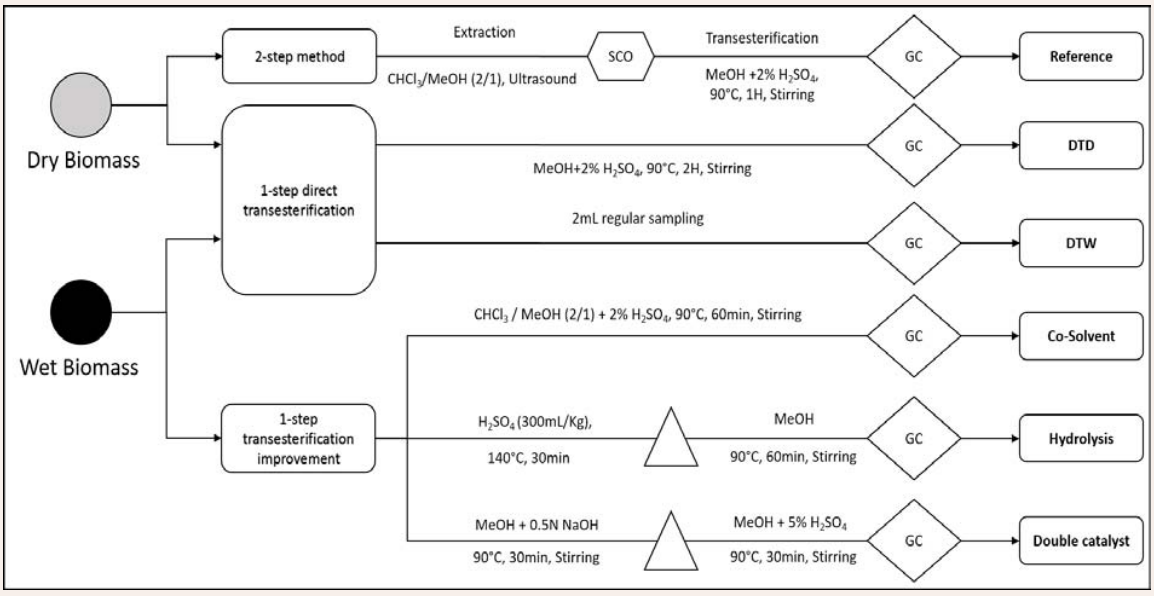

Figure 1 Schematic representation of the six different transesterification methods applied to dry and wet biomass of A. niger and *Y. lipolytica*. DTD: Direct Transesterification on Dry biomass; DTW: Direct Transesterification on Wet biomass; SCO: Single Cell Oil; GC: Gas Chromatography.

retention times with those of pure commercial standards (Sigma, USA) and concentration of each FAME was quantified using the C17:0 methyl ester as an internal standard. Example of typical chromatogram obtained is represented in Figure 2.

Statistical analysis

Statistical analysis of the results obtained was performed using the software XLSTAT Version 2015.5.01.23106 (AddInsoft). All experiments were conducted in duplicate. Data are expressed as mean ± standard error. The significant differences between the various methods of transesterification used were assessed using one-way analysis of variance follow by the Duncan method for paired comparisons at a confidence level of 95 %. "A-D classes" represent the ranking of the results according to this test. Results in the same class are not significantly different from each other whereas results in different classes are significantly different at 95% confidence interval.

RESULTS AND DISCUSSION

Results allowed us to select UAM as the reference method for the two-step lipid extraction (Supplementary Data). UAM is known to enhanced lipid extraction from algae because of cavitation [18,19], a phenomenon that releases large amounts of energy over small regions, at multiple locations [20]. The different behaviour of the fungi *A. niger* using ultrasound is probably due to the mycelial nature of the biomass and the mechanics of mass transfer in the Soxhlet method [20].

Determination of the optimal transesterification time

In order to evaluate the optimal reaction time, sampling of the reaction mixture was performed at regular intervals during the methanolysis of dry or wet biomass. The results, representing the sum of all the quantified FAMEs (based on the sum of all the FAMEs peaks areas), are shown in Figure 3. The optimal reaction time varies according to the microorganism and the moisture content considered. *A. niger* biomass (Figure 3A), showed the maximum fatty acid production at 120 minutes with a FAMEs production on dry biomass of 146 % of the value obtained with the two-step process (normalized to reference, NTR). A maximum value of 119 % NTR was attained from wet biomass after 90 minutes. Considering *Y. lipolytica* (Figure 3B), a maximum of 190 % NTR FAMEs production was obtained after 120 minutes of reaction with dry biomass although the reaction was almost finished after 60 minutes. The direct transesterification with wet biomass of *Y. lipolytica* was slower and less efficient, with a maximum FAMEs production of 143 %NTR after 120 minutes of reaction. It can be noted that methanolysis of dry biomass is already well advanced after only 30 minutes reaction time with 108 %NTR FAMEs production whereas the reaction with wet biomass at this time point barely achieved 52 %NTR FAMEs production. The direct transesterification of biomass without an extraction step gave higher FAMEs production than the commonly used two-step extraction-methanolysis procedure for both the yeast and the filamentous fungi, regardless of their moisture content. Higher FAMEs production in direct acid methanolysis of dry biomass has already been reported for microalgae [21], with a 148% increase in FAMEs production compared with a extraction/transmethylation process, and for yeast [22], with a maximum yield of 95 % of lipid content converted into FAMEs as compared with 13.3 % for conventional extraction/transmethylation process. Comparing the direct transesterification method with the Folch [23] and Bligh and Dyer [24] methods on wet microalgal biomass [25], it was shown that the Folch method yielded 77–93 % of the FAMEs production obtained by direct transesterification whereas the Bligh and Dyer method achieved only 19–63 %. This enhancement of FAMEs production by direct methanolysis has been attributed to the better cell disruption ability of direct methanolysis [26] and the reduction of losses due to the smaller number of steps in the process [25]. However,

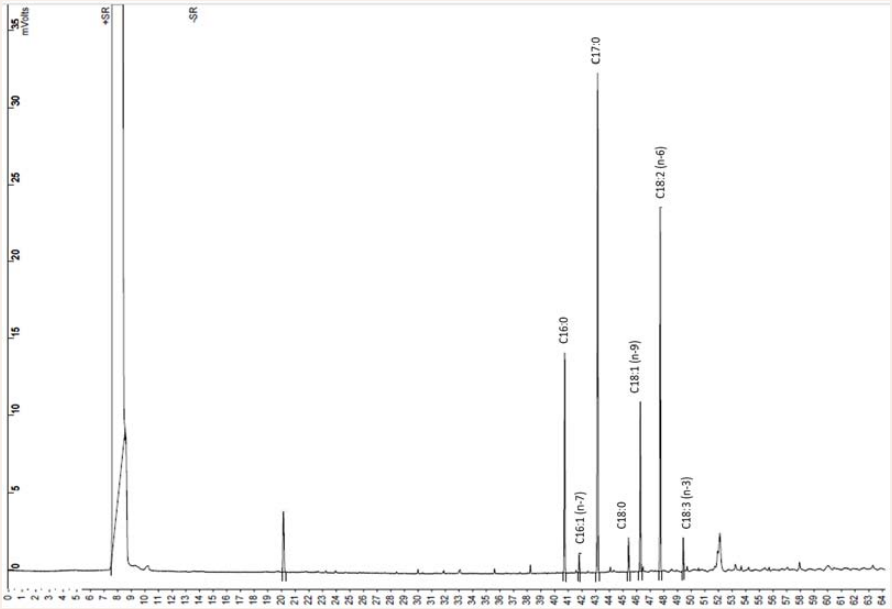

Figure 2 Typical chromatogram obtain by gas chromatography analysis. The figure illustrates elution of methanolysis product from direct transesterification of dry biomass of *A. niger*.

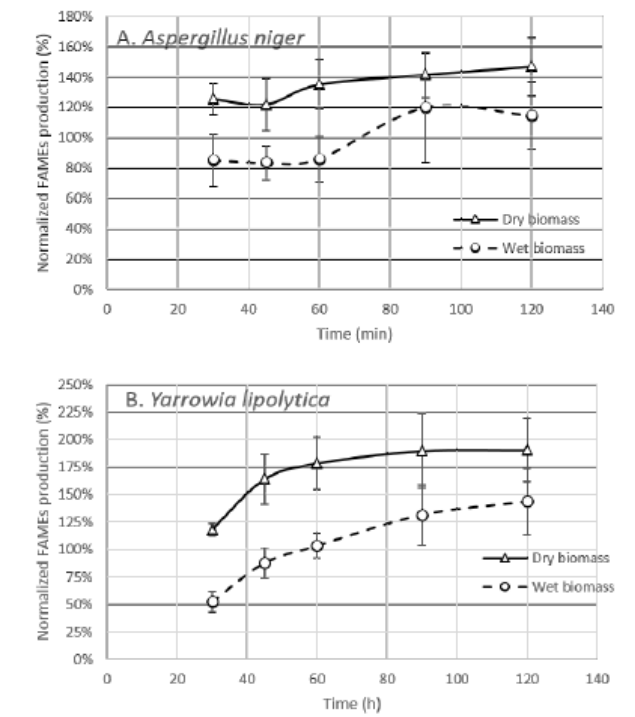

Figure 3 Plots of the FAMEs production by direct methanolysis for the dry and wet biomass of (A) *Aspergillus niger* and (B) *Yarrowia lipolytica*, normalised to the two- step extraction/esterification, versus the reaction time. Normalised FAMEs production (%) was calculated as percentage of the FAMEs production by the Reference two-step method of choice, for each microorganism.

direct methanolysis of wet biomass resulted in lower FAMEs production than direct methanolysis of dry biomass. This phenomenon has already been described [27]. In our case, the gap between dry and wet biomass was about 25 %. The only difference between the two methodologies is the water content of the biomass; hence this parameter is probably the cause of the decrease. Different methodologies were tested in order to reduce the gap between the yield of direct transesterification on dry and wet biomass, and are reported in the following parts of this study. As the reactions were almost complete after 60 minutes reaction time, the improvement methods on the following experiments were carried out using this reaction time.

Effect of different methods on transesterification of wet biomass

The present study focused on three possible modifications of the methanolysis process. The results in Figure 4 indicate that the positive effects of the treatments are weak. The use of chloroform as a co-solvent significantly reduced the FAMEs production to less than 50 % NTR for the two microorganisms considered. Hydrolysis of the biomass prior to methanolysis did not result in an improvement in FAMEs production compare to direct transesterification of the wet biomass in the case of *A. niger* biomass, and even reduced the reaction yield in the case of *Y. lipolytica*. The use of the double catalyst method on wet *Y. lipolytica* biomass showed a slight improvement in the FAMEs yield of 20 % compared with direct transesterification on wet biomass. The negative effect of water on the transesterification reaction is well established. Water may interfere with the reactions since it is a stronger electron donor than methanol [28]. Moreover, the presence of water in the biomass interferes with the catalyst, reducing the overall reaction efficiency. Fatty acids are saponified by alkaline catalysts in the presence of water, which hinders effective transesterification [25]. Using acid catalysts in the presence of water causes the acid catalyst to bind to water, leading to its deactivation [29]. The use of a co-solvent in which oil and methanol are miscible is a common practice for enhancing the reaction yield [30]. In our case, we investigated the possible improvement by the addition of chloroform to the wet biomass. As chloroform and water are immiscible, the moisture content of the biomass probably limits mass transfer from the intracellular medium to the reaction mixture, thus preventing lipid transfer to the organic phase and its methanolysis.

Hydrolysis of biomass prior to esterification is thought to be beneficial for FAMEs production from wet biomass because it can release free fatty acids from triglycerides, which are easier to methylate in aqueous solution using acid catalysts [14]. However, high concentrations of acid or long hydrolysis times and temperatures can have negative effects by reducing the amount of recoverable free fatty acids from wet microalgal biomass [14] and the total FAMEs of dry yeast biomass [31] or wet microalgal biomass [8]. In the two latter cited studies, acid was used as a catalyst and showed negative effects on FAMEs production at concentrations as low as 10.5 % (v/v) acetyl chloride at 100°C with wet *Nannochloropsis gaditana* [8] or 0.4 mol/l H_2SO_4 at 70°C with dry yeast [31]. In our case, we used the harsher conditions of concentrated sulphuric acid at 140°C as a hydrolysis pretreatment for wet biomass. The decrease in FAMEs content observed with the yeast *Yarrowia lipolytica* could be due to the hydrolysis conditions being too harsh for this yeast, leading to a reduction in the fatty acids and recoverable FAMEs, hence necessitating a careful adjustment of the reaction time and concentration. A detrimental effect of excess acid on the total extractable FAMEs is a reasonable hypothesis because side reactions such as acid-promoted polymerisation might occur in harsh conditions for unsaturated fatty acids with one or more double bonds [8]. The

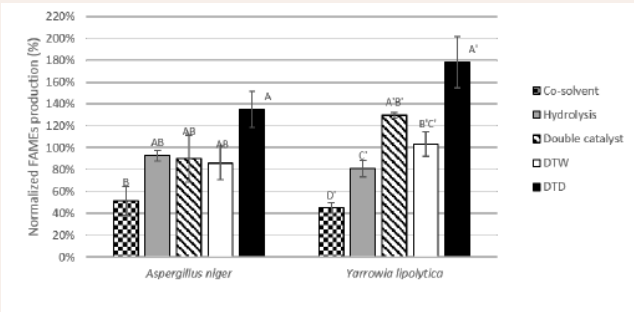

Figure 4 Plot of the normalized FAMEs production from wet biomass and dry biomass, by different esterification procedures, for A. niger and *Y. lipolytica* Normalised FAMEs production (%) was calculated as percentage of the FAMEs production by the Reference two-step method of choice, for each microorganism A–C represent significantly different classes for A. niger biomass. A'–D' represent significantly different classes for *Y. lipolytica* biomass.

difference in behaviour between *A. niger* and *Y. lipolytica* could be due to differences in the resistance of the cells to the acid. For instance, the cell wall composition of *A. niger* differs from that of *Y. lipolytica* principally in α- glucan content [32,33], which could contribute to better preservation of the filamentous fungus cells compared with the yeast cells during hydrolysis. Double catalyst use has been studied in order to combine the advantages of both types of catalyst. Alkaline catalysts offer the possibility of enhances the rate of triglyceride ransesterification [34] whereas acid catalysts improve the overall reaction, esterifying free atty acids and phospholipids [27]. The synergistic effect of the two catalyst types has already been recognised using pure glyceryl-triheptadecanoate with water addition, leading to a 100% conversion with double catalyst as compared to a 32% conversion for acid catalyst alone, using a complex wet microalgal matrix [25]. These authors suspected that the use of a basic catalyst in the presence of water resulted in saponification and cleavage of ester bonds between fatty acids and glycerol. This removed the water and allowed the acid catalyst to esterify the free fatty acids at a faster rate. An improvement as high as 462.6 % compared with the Bligh and Dyer methodology has been claimed using dry biomass of microalgae after optimisation with the sequential use of basic catalyst followed by acid catalyst [13]. Based on field emission scanning electron microscopy, they proposed that the order of catalyst use is important, as the alkaline catalyst provides greater destabilisation of microalgal cells compared with the acid catalyst, liberating more oil. The ability of the acid catalyst to convert triglycerides, free fatty acids and other complex lipids then enhanced the amount of FAMEs produced [34].

Our results are far from the results reported earlier [13]. We used wet biomass of a yeast and a fungus instead of dry biomass of microalgae, and these results show that the optimized parameters are dependent on the biomass used. However, these results are still encouraging and it is possible that the process can be improved. The FAMEs composition of the transesterification products for the two biomass types tested in each modified process is represented in Figure 5.

The major FAMEs detected in the *A. niger* biomass (Figure 5A) using the reference method were linoleic acid methyl ester (C18:2 n-6; LAME), oleic acid methyl ester (C18:1 n-9; OAME) and palmitic acid methyl ester (C16:0 ; PAME), that collectively contributed to 86.6 % of total FAMEs pool. PUFA were found to be the major FA class (46.7 %), followed by MUFA (33, 3 %) and SFA (19,7 %). The high proportions of LAME, OAME and PAME are in agreement with previous analyses for *A. niger* biomass. Linoleic, oleic and palmitic acids were also found to be the major FAs in *A. niger* biomass grown on commercial sugar, corresponding to 44.2%, 34.2% and 12.6% of total FAs, respectively [35]. The FAMEs profile of *A. niger* biomass was quite similar after growth on bagasse, displaying values of 53.5 % LAME, 25.5 % OAME and 7.2 % PAME [36]. In the case of lipids extracted from the *Y. lipolytica* biomass with the reference method (Figure 5B), MUFA were found to be the major FA class (40.7 %), followed by SFA (29.9 %) and PUFA (29.1 %). FAMEs profile was characterized by 33.6 % of OAME, 25.0 % of LAME and 22.7 % of PAME. The fatty acids composition of *Y. lipolytica* is known to be dependent on the carbon source [37]. When grown on glycerol, the major fatty acids were OAME and LAME with 47 % and 21 %, respectively. PAME

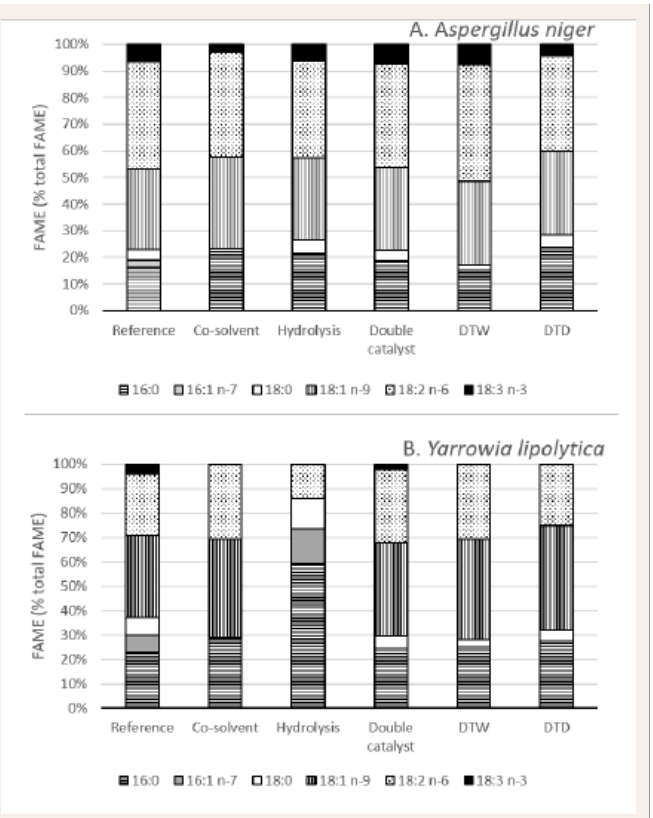

Figure 5 Percentage composition of the FAMEs production by different esterification methods for A. niger and *Y. lipolytica* 16:0 = methyl ester (ME) of the hexadecanoic acid; 16:1 (n-7) = ME of the *cis*-9 hexadecenoic acid; 18:0 = ME of the Octadecanoic acid; 18:1 (n-9) = ME of the *cis*-9 octadecenoic acid; 18:2 (n-6) = ME of the *cis*-9, *cis*-12 octadecadienoic acid; 18:3 (n-3) = ME of the *cis*-9, *cis*-12, *cis*-15 octadecatrienoic acid.

and SAME represented 15 % and 13 %, respectively [38]. Growth on distillery spent wash modified the oil composition of the yeast biomass, reducing the polyunsaturated fatty acids (PUFA), principally LAME by a factor of two. There was 20 % more MUFA and a two-fold increase in SFA after growth on vinasse compared with results reported elsewhere [39].

Comparison of each methodology for the two types of biomass revealed that the co-solvent method resulted in selectivity against OAME, as shown by the low yield of OAME from the two biomass types with the co-solvent method compared with the reference method. Moreover, no direct method was able to extract PoAME from *A. niger* biomass, leading to an underestimation of its content. The only wet methodology that could extract ALnAME from *Y. lipolytica* wet biomass was the double catalyst method. This observation, along with the improvement in yield observed in (Figure 4), supports the hypothesis that the double catalyst strongly destabilizes the biomass, resulting in increased lipid release and a wider variety of esterified molecules than with the other methods tested. The hydrolysis method greatly impacted both the total FAMEs extracted from *Y. lipolytica* wet biomass, and the composition of the FAMEs extracted. It probably oxidized the polyunsaturated fatty acids [40] as reflected in the increase in SFA content to over 70 % of the total fatty acids and the decrease in the other two categories. Only hydrolysis was able

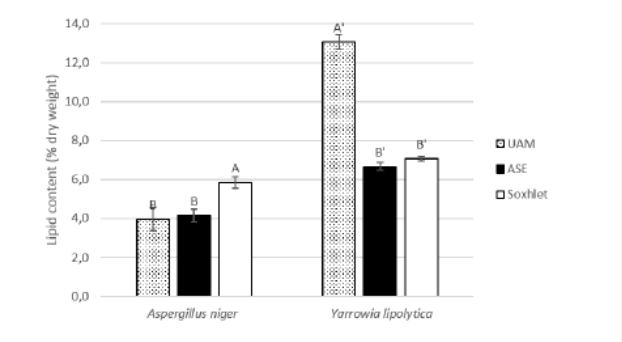

Figure 6 Comparison of three different lipid extraction methods on fungal and yeast grown on sugarcane distillery spent wash. A and B represent significantly different classes for A. niger biomass. A' and B' represent significantly different classes for *Y. lipolytica* biomass.

to extract PoAME from *Y. lipolytica* wet biomass. This method also reduced the extractable FAMEs as shown by the decrease in the total FAMEs content (Figure 4). The reduction in the total FAMEs produced eventually resulted in over-representation of particular classes of fatty acids, explaining the apparent high content of PoAME and SAME compared with the other methods tested. Careful attention should be paid to the acid addition when dealing with such yeasts grown on distillery spent wash, and the conditions should be optimised prior to its use. It is worth mentioning that the PUFA content of the vinasse-grown biomass is high, representing 46 % and 29.1 % of the FAMEs, respectively for *A. niger* and *Y. lipolytica*. From a biofuel viewpoint, high PUFAs improve the cold flow properties of the energetic fuel produced but also hinder its oxidative stability. MUFA are considered as the best compromise with regard to oxidation stability and cold flow properties. Since the FAMEs profiles obtained using vinasses contained moderate amounts of PUFA, it could be considered a good medium for energetic lipid production.

CONCLUSIONS

The main purpose of this work was to assess different methodologies of direct wet lipid methanolysis of *Aspergillus niger* and *Yarrowia lipolytica* biomass grown on sugarcane distillery spent wash. Three methodologies were compared to two-step ultrasound assisted extraction of lipids followed by methanolysis of the extract and direct transesterification of dry or wet biomass. Direct transesterification methods resulted in a higher yield of FAMEs compared with the two-step method, regardless of the moisture content of the biomass, although dry biomass gave higher yields than wet biomass. The double catalyst methodology showed an improvement in the quality and quantity of fatty acids extracted from wet *Y. lipolytica* biomass compared with the other tested processes, showing that improvements can be obtained. Hydrolysis of the biomass prior to methanolysis deeply affected the FAMEs composition of the yeast tested, underlying the necessity of precisely controlling the reaction parameters.

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SUPPLEMENTARY DATA

Choice of the two-step reference extraction method

Different extraction methods were studied in order to choose a reference two-step transesterification method namely Soxhlet extraction, ultrasound assisted maceration (UAM) and accelerated solvent extraction (ASE). The parameters for each method are shown in table below.

Table 1: Extraction parameters for two-step methods.

Two-step methods	Conditions
UAM	45 kHz; 80 % amplitude; 40 °C; 3x15 minutes,
ASE	100 °C; 3x10 min static cycles; 0.97-1.38 MPa; 100 % flush, 120 sec purge
Soxhlet	Boiling; 5 h

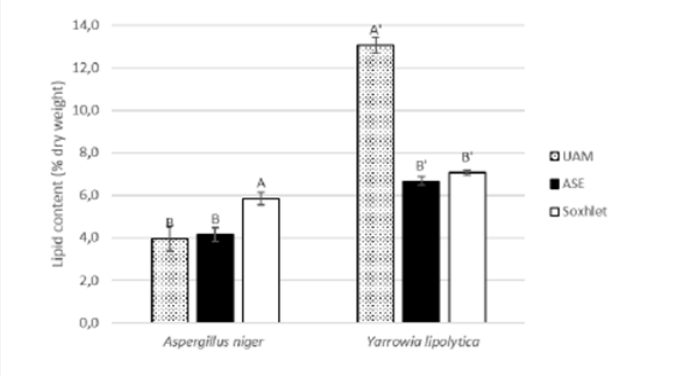
Freeze-dried biomass from the same cultivation batch was crushed using a mortar and pestle and used for comparison of the extraction methods using mixture of chloroform: methanol (2:1, v/v) as the solvent.

Ultrasound assisted maceration (UAM): freeze-dried biomass (100 mg) was mixed with 10 ml extraction solvent and sonicated (Transsonic TI-H-10) at 45 kHz, 40 °C and 80 % ower for 15 min. Chloroform:methanol (2:1 v/v) mixture (3 ml) and distilled water (3 ml) was added for phase separation before recovery of the organic layer. Two further cycles of solvent extraction were performed.

Accelerated solvent extraction (ASE): freeze-dried biomass (700 mg) was mixed with Fontainebleau sand to fill a 10 ml stainless steel ASE extraction cell (ASE 350, Dionex). The biomass underwent three successive 10 min-static cycles at 100 °C with the extraction solvent using a 5 min heat-up time. A pressure of 0.97–1.38 MPa was applied. The flush volume was 100 % of the extraction cell volume. The extract was purged from the sample cell using pressurised nitrogen for 120 s. Water (10 ml) was then mixed with the extracted mixture. The organic layer was recovered after phase separation.

Soxhlet extraction: freeze-dried biomass (700 mg) was extracted in a cellulose bucket and subjected to Soxhlet treatment with 100 ml extraction solvent for 5 h at boiling point. The treated extract was then mixed with 30 ml water for phase separation. For each method, the lipid content of the biomass was evaluated gravimetrically after drying with anhydrous Na_2SO_4.

The results in figure below



show a significantly higher 41 % increase in lipid extraction by the Soxhlet method compared with ASE for A. niger. On the contrary, UAM gave the best results with Y. lipolytica, with an 84 % higher lipid content than the Soxhlet method. UAM is known to enhanced lipid extraction from algae because of the cavitation phenomenon. The different behaviour of the fungi A. niger using ultrasound is probably due to the mycelial nature of the biomass and the mechanics of mass transfer in the Soxhlet method. In the Soxhlet method, oil is recovered over 5 hours by simple diffusion through the cell membrane and the lipids always diffuse into fresh solvent, whereas the solvent was only renewed three times in the three 15 minute steps of the UAM protocol. Moreover, UAM efficiency is known to decrease with increasing cell density. The highly dense and intricate nature of mycelia could therefore offer greater protection from cavitation bubbles compared with unicellular yeast and the limited time of UAM extraction may have contributed to a decrease in the transfer of lipids from the mycelium to the solvent, leading to better results with Soxhlet extraction than with UAM. Since the difference between UAM and Soxhlet is smaller for A. niger than for Y. lipolytica, UAM was chosen as the reference method for the two-step lipid extraction.