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Article

Sugarcane Distillery Spent Wash, a New Resource for Third-Generation Biodiesel Production

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Abstract: Industrial production of biodiesel from microbial catalysts requires large volume of low-cost feedstock for lipid production. Vinasse, also known as distillery spent wash (DSW), is a liquid waste produced in large amounts by ethanol distilleries. This effluent is particularly rich in organic matter, and may be considered as a potential resource for the production of fungal lipids. The present study aimed at evaluating the potential of vinasse from a distillery located in Reunion Island for yeast and fungal growth, lipid production, and suitability for biodiesel requirements. Among the 28 different strains tested, we found that *Aspergillus niger* grown on pure vinasse allowed biomass production of up to 24.05 g/L (dry weight), whereas *Aspergillus awamori* produced the maximum amount of lipid, at 2.27 g/L. Nutrient removal and vinasse remediation were found to be the best for *A. niger* and *Cryptococcus curvatus*, reaching a maximum of 50% for nitrogen, and *A. awamori* showed 50% carbon removal. Lipids produced were principally composed of C16:0, C18:1 (n-9), and C18:2 (n-6), thus resembling the vegetal oil used in the biodiesel production. This work has shown that vinasse can support production of biomass and lipids from fungi and yeast suitable for energetic use and that its polluting charge can be significantly reduced through this process.

Keywords: vinasse; sugarcane distillery spent wash; single cell oil; biodiesel; oleaginous microorganisms

1. Introduction

Global reserves of fossil oil are depleting, resulting in a greater consciousness of the necessity to develop new sources of renewable fuels. For this purpose, industrial production of biodiesel from oleaginous plants, animal fat, or waste oils from the food industry has already been implemented in several countries [1,2]. However, fuels derived from edible vegetal resources, also known as first-generation biofuels, have been severely criticized because of many related problems arising from competition with the food industry [3]. Other sources of lipids have been investigated, including non-edible vegetal resources such as Jatropha [4,5], insect larvae [6,7], or microbial oil. Microbial oil, or single cell oil (SCO), produced by oleaginous microorganisms including bacteria, yeasts, filamentous fungi, and microalgae, are considered as a potential source of oil for biodiesel production [8–11]. Indeed, they can accumulate large quantities of oil inside lipid bodies, with a composition of fatty acids similar to that of plant-based oil [12–14]. The advantages of microorganisms over other biological sources of lipids are their ability to metabolize complex mediums such as industrial wastes, and their rapid growth rate, which enables a reduction in production costs and pollution [13–19]. However, common industrial wastes used for microbiological production such as molasses are already extensively used.

Third-generation biofuels will need new resources that can support large volumes of production at a minimal cost.

Distillery spent wash (DSW), also called vinasse, is the waste stream from distilleries. It is produced annually in large volumes, representing 12 to 15 times the alcohol volume produced during the distillation step [20]. The world production of alcohol is expected to grow, partially due to the production of bioethanol for energetic purposes, resulting in a strong increase in vinasse production causing the pollution of large natural areas [21]. Research on DSW is mainly focused on the pollution issue, namely on physical and anaerobic biological treatment [21–23]. Anaerobic valorisation of this industrial waste for methane production is already the main treatment applied to DSW, highlighting its potential for microbial production [21]. However, gaseous fuel are less easy to handle and possess lower energetic density than liquid fuels. Fungi and yeasts are ubiquitous microorganisms able to degrade a large molecule spectrum, including industrial wastewater. Some of them are also able to accumulate SCO at non-negligible quantities, representing a potential feedstock for biodiesel production [24–26]. Thus, a novel integrated route for vinasse management using fungi and yeasts for SCO production could open the way for third-generation biodiesel.

This study aims at evaluating the potential of DSW as a culture medium for oleaginous fungi and yeasts, assessing the depolluting effect of their growth, determining the lipid production and composition of this nutritive source, and estimating the characteristics of the produced biodiesel.

2. Materials and Methods

2.1. Strains and Culture Conditions

Twenty-eight yeast and fungal strains were obtained from the Mycothèque de l'Université Catholique de Louvain (MUCL) and are listed in Table 1. Upon reception, strains were revivified on storage medium, namely YPDA medium for yeasts (yeast extract 10 g/L, peptone 20 g/L, dextrose 20 g/L, agar 15 g/L) and PDA medium for filamentous fungi (potato dextrose agar from BD Difco, New Jersey, USA) and kept at 4 °C until use.

Table 1. Yeast and filamentous fungi strains used in this study and lipid contents described in literature (when available).

Name of the Strain	Accession Number	Lipid Content (% Dry Weight)	References
Absidia corymbifera	MUCL 10046	20	[27,28]
Aspergillus amstelodami	MUCL 14404	51.1	[29]
Aspergillus awamori	MUCL 717	28.3	[30]
Aspergillus niger	MUCL 19001	-	-
Aspergillus oryzae	MUCL 19009	40	[16]
Aspergillus repens	MUCL 3653	18.18	[29]
Aspergillus terreus	MUCL 38811	54	[31]
Candida curvata	MUCL 27713	37.7	[32]
Candida tropicalis	MUCL 29893	46.8	[33]
Cryptococcus albidus	MUCL 30400	27	[34]
Cunninghamella echinulata	MUCL 38718	57.7	[35]
Gibberella fujikuroi	MUCL 31591	-	-
Lipomyces starkeyi	MUCL 39773	68	[36]
Mortierella vinacea	MUCL 15067	-	-
Mucor circinelloides	MUCL 9662	46	[37]
Penicillium spinulosum	MUCL 11940	-	-
Pichia angusta	MUCL 27761	34.9	[38]
Pichia guilliermondii	MUCL 29837	48.6	[39]
Pichia kudriavzevii	MUCL 29849	23	[40]
Rhodosporidium toruloides	MUCL 27808	58.3	[41]
Rhodotorula glutinis	MUCL 27808	36.5	[42]
Rhodotorula graminis	MUCL 11961	54	[43]
Rhodotorula mucilaginosa	MUCL 11918	48.6	[44]
Schizophyllum commune	MUCL 30748	-	-
Thamnidium elegans	MUCL 15507	71.1	[12]
Trichoderma viride	MUCL 29749	-	-
Trichosporon fermentans	MUCL 14474	62.4	[45]
Yarrowia lipolytica	MUCL 30108	36	[46]

Before cultivation, strains were revived at 25 °C on the corresponding storage medium until colony formation. Yeast cells and fungal spores were resuspended in 5 mL of NaCl 9 g/L and 5×10^5 cells were pipetted to inoculate 50 mL of sterile crude DSW in a 250-mL flask. Flasks were incubated in an orbital shaker at 25 °C, 110 rpm, for 10 days.

Samples of crude DSW (sugarcane campaign of 2013) were collected directly at the distillation column outlet of *Distillerie Rivière du Mât*, Saint-Benoit, Reunion Island. After cooling, DSW was stored in small sterile bags at −20 °C until use. Prior to culturing, DSW was thawed and sterilized by autoclaving at 121 °C for 20 min. Specific physicochemical characteristics of DSW used in this work are presented in Table 2.

Table 2. Characteristics of distillery spent wash (DSW) from Distillerie Rivière du Mât, Reunion Island (COD: chemical oxygen demand; TC: total carbon; TN: total nitrogen; C/N: carbon/nitrogen ratio; TSS: total suspended solids).

Parameters	Concentration or Value
pH	4.80 ± 0.07
COD (g L^{-1})	76.30 ± 4.84
TC (g L^{-1})	28.80 ± 1.87
TN (g L^{-1})	1.48 ± 0.13
C/N	19.5
TSS (g L^{-1})	8.50 ± 1.33

2.2. Biomass Determination

After cultivation, 40 mL of broth were harvested and treated as follow: for yeast, cells were placed in a pre-weighed conical tube and biomass was concentrated by centrifugation at 7500 rpm during 15 min at room temperature on a Heraeus Biofuge PrimoR (Thermo Fisher Scientific, Darmstadt, Germany) with a Fixed Angle Highconic rotor. For filamentous fungi, cells were recovered by filtration over a Buchner apparatus on Whatman No. 1 filter (porosity 11 μm). Biomasses were rinsed twice with distilled water and frozen at −80 °C overnight. Frozen biomass was then lyophilized with a Labconco FreeZone 2.5 apparatus for 2 days. Tubes were weighed after freeze-drying to determine biomass production.

2.3. Total Lipids Extraction

Total lipid extraction was performed according to the ultrasound assisted maceration method recently described [14]. Freeze-dried cells were broken using a mortar and 100 mg of the crushed cells were recovered to determine the lipids content. Then, 10 mL of a chloroform/methanol mixture (2:1 v/v) were added to the crushed cells in a glass tube. The suspension was thoroughly mixed for 1 min and then treated in an ultrasonic bath (Transsonic TI-H-10) at 45 kHz, 40 °C for 3 cycles of 5 min. Then, 3 mL distilled water were added to 3 mL of the chloroform/methanol mixture, mixed thoroughly for 1 min, and centrifuged at 2000 rpm for 5 min to facilitate the separation of the phases. The lower organic layer was harvested in a glass beaker. The biomass at the interface was again extracted twice using the same extraction steps. The lower organic layers from the three extraction steps were pooled together and dried over anhydrous Na_2SO_4, and filtered and concentrated using a rotary evaporator (Buchi, Switzerland). Concentrated lipids were then transferred to a pre-weighed vial, evaporated overnight and kept at 4 °C until use. Dry vials were weighed to determine the mass of lipids extracted and the lipid content of the biomass (Equation (1)).

$$\text{Lipid content} = \text{mass lipids}/\text{mass biomass}, \quad (1)$$

2.4. Monitoring of the Growth Kinetics of the Selected Strains

Among the 28 strains, the two strains with maximum biomass production (*Candida curvata* and *Aspergillus awamori*), two others exhibiting maximum lipid content (*Rhodosporidium toruloides* and *Mortierella vinacea*) and two strains of industrial interest (*Yarrowia lipolytica* and *Aspergillus niger*) were selected. The growth characteristics of these six strains were monitored more precisely in controlled fermentor. The three yeast strains were cultivated during 10 days in a 2 L Biostat B+ (Sartorius Stedim Biotech GmbH, Göttingen, Germany) bioreactor containing 1.5 L of crude autoclaved DSW. The fermentor was inoculated with 50 mL of yeast preculture grown during 72 h in crude DSW. Temperature was maintained at 28 °C and pO2 was kept above 50% varying the agitation speed. pH of the broth and dissolved oxygen were monitored using an Easyferm plus K8 200 (Hamilton Company, Bonaduz, Switzerland) pHmeter and an OxyFerm FDA 225 (Hamilton). Then, 10-mL aliquots were harvested at different sampling times and biomass was determined as described previously. Samples of liquid medium were frozen at −20 °C until use. Because fungal cells produced heterogeneous broth, the growth characteristics of the 3 fungal strains were determined during growth in shacked flasks. Ten identical flasks containing 50 mL crude DSW and inoculated with 10^5 spores each were incubated at 28 °C and 150 rpm in an orbital shaker for 10 days. One flask was used every 24 h to evaluate the biomass content and a sample of the liquid medium was frozen at −20 °C for further analysis.

2.5. Analytical Methods

Transesterification step was performed according to Liu et al. [47]. 5 mL of 2% H_2SO_4/methanol (*v*/*v*) was added to the extracted lipids. The mixture was reflux heated at 70 °C during 1 h under constant stirring. The flasks were then let to cool down at room temperature and 2 mL of hexane and 0.75 mL of distilled water were added to the mixture. The two phases were allowed to separate and the upper hexane layer was recovered and dried over anhydrous $MgSO_4$.

Analysis of the fatty acid composition was carried out on a CP3800 Gas chromatograph (Varian, Santa Clarita, CA) equipped with a SG BPX-70 capillary column and a flame ionization detector. Operating conditions were set as follows: 240 °C injector temperature, 260 °C detector temperature, 1.3 mL/min flow rate and oven temperature programmed from 120 °C to 230 °C at 3 °C/min and then 230 °C for 17 min. Then, 0.5 µL of transesterification product was injected and subjected to gas chromatograph along with flame ionized detector (GC-FID) analysis. The initial split ratio was set at 1:5 and after 5 min of elution, analysis was run using a 1:50 split ratio. The percentage of the peak area was assumed to be the percentage content of the corresponding compound.

Chemical oxygen demand (COD), total nitrogen (TN), and total organic carbon (TC) were determined using commercial kits LCK914 for COD, LCK 338 for TN and LCK 387 for TOC from Hach Lange. The concentration of total suspended solids (TSSs) in the broth medium was determined according to [19]. The properties of transesterification products were calculated using their fatty acid methyl ester (FAME) composition and using equations proposed by Ramirez-Verduzco et al. [48] for cetane number (CN), density (Den) and higher heating value (HHV). The cold filter plugging point (CFPP) was estimated using equations from Ramos et al. [49]. Viscosity (Vis) was estimated using an equation from Su et al. [50].

2.6. Statistical Analysis

Statistical analysis of the results obtained were performed using the software XLSTAT Version 2015.5.01.23106 (AddInsoft, Paris, France). Screening cultures were conducted in triplicate and kinetics were conducted in duplicate. Data are expressed as mean ± standard error. The significance of differences between treatments and their respective control were assessed through a Student test with a 95% signification level, whereas multiple comparisons were assessed through one-way analysis of variance followed by Duncan method for pair comparisons at a confidence level of 95%.

3. Results and Discussion

3.1. Screening for Microbial Growth and Lipid Accumulation on Crude DSW

Thirteen yeast and fifteen fungal strains were selected for their ability to produce high amount of lipids on lipogenic media or for their ability to grow on crude DSW. The results of the screening for biomass production and lipid accumulation on crude DSW media are presented in Table 3.

Table 3. Total dry biomass, lipid content and lipid yield of fungal and yeast strains grown on crude distillery spent wash (DSW) for 10 days.

Strains	Total Dry Biomass (g/L)	Lipid Content (% dcw)	Lipid Yield (g/L)
Fungal strains			
M. vinacea	14.30 ± 0.77	15.84 ± 0.07	2.27 ± 0.12
A. awamori	19.16 ± 2.36	10.96 ± 0.5	2.08 ± 0.18
A. niger	24.06 ± 2.2	6.94 ± 0.33	1.66 ± 0.09
M. circinelloides	10.48 ± 0.2	14.06 ± 0.41	1.47 ± 0.03
T. elegans	12.36 ± 0.48	11.59 ± 1.5	1.42 ± 0.16
A. amstelodami	13.51 ± 0.66	9.95 ± 0.36	1.35 ± 0.11
A. corymbifera	9.18 ± 0.34	10.10 ± 0.81	0.93 ± 0.09
C. echinulata	7.24 ± 1.16	11.39 ± 0.4	0.82 ± 0.13
A. repens	6.40 ± 1.37	12.66 ± 0.1	0.81 ± 0.18
A. oryzae	13.3 ± 0.84	6.13 ± 0.25	0.81 ± 0.03
P. spinulosum	8.13 ± 0.46	9.61 ± 0.17	0.78 ± 0.05
S. commune	5.32 ± 2.03	10.65 ± 0.28	0.56 ± 0.2
T. viride	4.97 ± 0.37	10.85 ± 0.34	0.54 ± 0.02
A. terreus	6.42 ± 3.45	4.39 ± 0.3	0.30 ± 0.16
G. fujikoroi	1.66 ± 1.66	4.63 ± 0.06	0.08 ± 0.08
Yeast strains			
C. curvata	8.95 ± 0.29	10.96 ± 0.19	0.98 ± 0.03
R. graminis	7.64 ± 0.44	11.00 ± 0.41	0.84 ± 0.03
R. mucilaginosa	7.80 ± 0.16	10.21 ± 0.52	0.80 ± 0.03
T. fermentans	6.20 ± 0.64	11.44 ± 0.63	0.70 ± 0.06
P. angusta	4.47 ± 0.36	14.83 ± 0.62	0.66 ± 0.03
R. glutinis	6.40 ± 1.13	8.21 ± 0.09	0.53 ± 0.1
C. albidus	4.54 ± 2.43	8.48 ± 0.93	0.43 ± 0.24
C. tropicalis	6.63 ± 0.39	5.73 ± 0.18	0.38 ± 0.03
R. toruloides	1.96 ± 0.14	18.15 ± 0.18	0.36 ± 0.02
P. guillermondii	4.92 ± 0.17	6.57 ± 0.29	0.32 ± 0.03
Y. lipolytica	3.27 ± 0.06	7.28 ± 0.24	0.24 ± 0.01
P. kudriavzevii	3.17 ± 0.08	7.28 ± 1.33	0.23 ± 0.04
L. starkeyi	1.22 ± 0.12	16.71 ± 1.41	0.20 ± 0.01

With respect to biomass production, the best biomass yields were obtained for fungal strains. Biomass production of *Aspergillus niger* and *A. awamori* in crude vinasse yielded respectively 24.06 g/L and 19.16 g/L dry weight (dw). Comparatively, the yeast strains produced globally much less biomass. The highest biomass production of the yeasts was found for *Candida curvata*, *Rhodotorula mucilaginosa*, and *Rhodotorula graminis* at 8.95 g/L, 7.80 g/L, and 7.64 g/L (dw) respectively. Among the yeasts, more than 50% of the strains produced less than 5 g/L of biomass.

With respect to lipid production, the best producing strains were also found within the filamentous fungi set of strains. *A. awamori* and *Mortierella vinacea* showed the highest lipid production yield, at 2.08 g/L and 2.27 g/L (dw), respectively. *A. niger*, which yielded the best biomass concentration, accumulated up to 1.66 g/L of intracellular lipids. Among the yeast strains, the highest production of lipids was found for *C. curvata*, *R. mucilaginosa*, *R. graminis*, and *Trichosporon fermentans*, at 0.98 g/L, 0.80 g/L, 0.84 g/L, and 0.70 g/L, respectively (Table 3).

Oleaginous microorganisms are characterized by their capacity to accumulate more than 20% of lipids (dw) when cultivated on a high C/N ratio medium, i.e., when the lipid metabolism is activated [46]. Although most of the microorganisms used in this study are considered as oleaginous

strains under our experimental conditions, the maximum lipid content was achieved for *M. vinacea* with 15.84% dw and for *R. toruloides* with 18.15% dw.

The vinasse used in this study had an initial C/N ratio of approximately 20 (Table 2) which is apparently still too low to stimulate lipid accumulation in this experiment. Moreover the chemical composition of DSW is highly complex with compounds such as melanoidins from Maillard reactions, caramels, and phenolic colorants [21] which might interfere negatively with microbial growth. Filamentous fungi are known for their ability to degrade complex substrates thanks to their large variety of extracellular enzymes and broad metabolism [24]. Thus, this ability might explain the higher biomass yield of filamentous fungi on crude vinasse as compared to the yeasts.

Gonzalez-Garcia et al. [51] reported that *Cryptococcus curvatus* and *Rhodotorula glutinis* produced respectively 5.19 and 6.06 g/L of biomass on pure distillery wastewater. Using our local vinasse, we obtained biomass production yields of 4.54 g/L, 6.4 g/L, 7.64 g/L, and 7.8 g/L, respectively for *Cryptococcus albidus*, *R. glutinis*, *R. graminis*, and *R. mucilaginosa* which were in good agreement with reported data from Gonzalez-Garcia et al. [51]. However, they obtained lipid production values of 1.49 g/L and 1.86 g/L, respectively for *C. curvatus* and *R. glutinis* after 144 h of culture on pure vinasse, whereas we found that the best lipid production from yeasts was 0.98 g/L for *C. curvata* after 240 h of culture.

Nitayavardhana et al. [52] studied the growth of the edible fungus *Rhizopus oligosporus* on DSW with nitrogen and phosphorus supplementation in an airlift bioreactor. In their experiments, the authors found a maximum fungal biomass production of 4.91 g/L with a 3.44% lipid content. Nahas et al. [53] also reported the growth of *A. niger* on diluted vinasse and reported a 25.5 g/L biomass production for a dilution 1/5. These results illustrated the diversity of filamentous fungi behaviours grown on DSW but corroborate our results, spanning from low biomass production at 5 g/L to very high biomass yield at 24 g/L. These studies also confirmed that filamentous fungi are more suitable for biomass and lipid production on pure DSW than yeasts.

3.2. Growth Kinetics of the Best Biomass or Lipid Producer Strains

Kinetics of biomass and lipid production and carbon and nitrogen removal from the medium of three fungal (*A. niger*, *A. awamori*, and *M. vinacea*) and three yeast (*C. curvata*, *R. toruloides*, and *Y. lipolytica*) strains were assessed during growth on pure vinasse (Figure 1).

Our results showed that a maximum biomass production was reached within the first 96 h of growth for the yeasts with 7.11 g/L, 10.14 g/L and 5.4 g/L for *R. toruloides*, *C. curvata*, and *Y. lipolytica*, respectively, whereas biomass production of the fungal strains occurred all along the culture, reaching a maximum biomass of 16.3 g/L, 21.4 g/L and 17.3 g/L for *M. vinacea*, *A. niger,* and *A. awamori*, respectively. With respect to lipid production, we observed only a slight increase of intracellular lipids all along the growth within the range of 1 to 2 g/L both for yeast and fungal strains.

DSW is a medium containing a significant amount of suspended solids (TSS) ranging from 5 to 12 g/L (8.5 g L^{-1} in our experiment, see Table 2). TSS are residues from the alcoholic fermentation such as residual yeast cells from the alcoholic fermentation step. The lipid content of raw DSW resulted from the yeast residual biomass initially present in the medium. These lipids are probably not consumed during the fermentation as they are found at the same level at the end of the culture and thus could contribute to the overall biodiesel production.

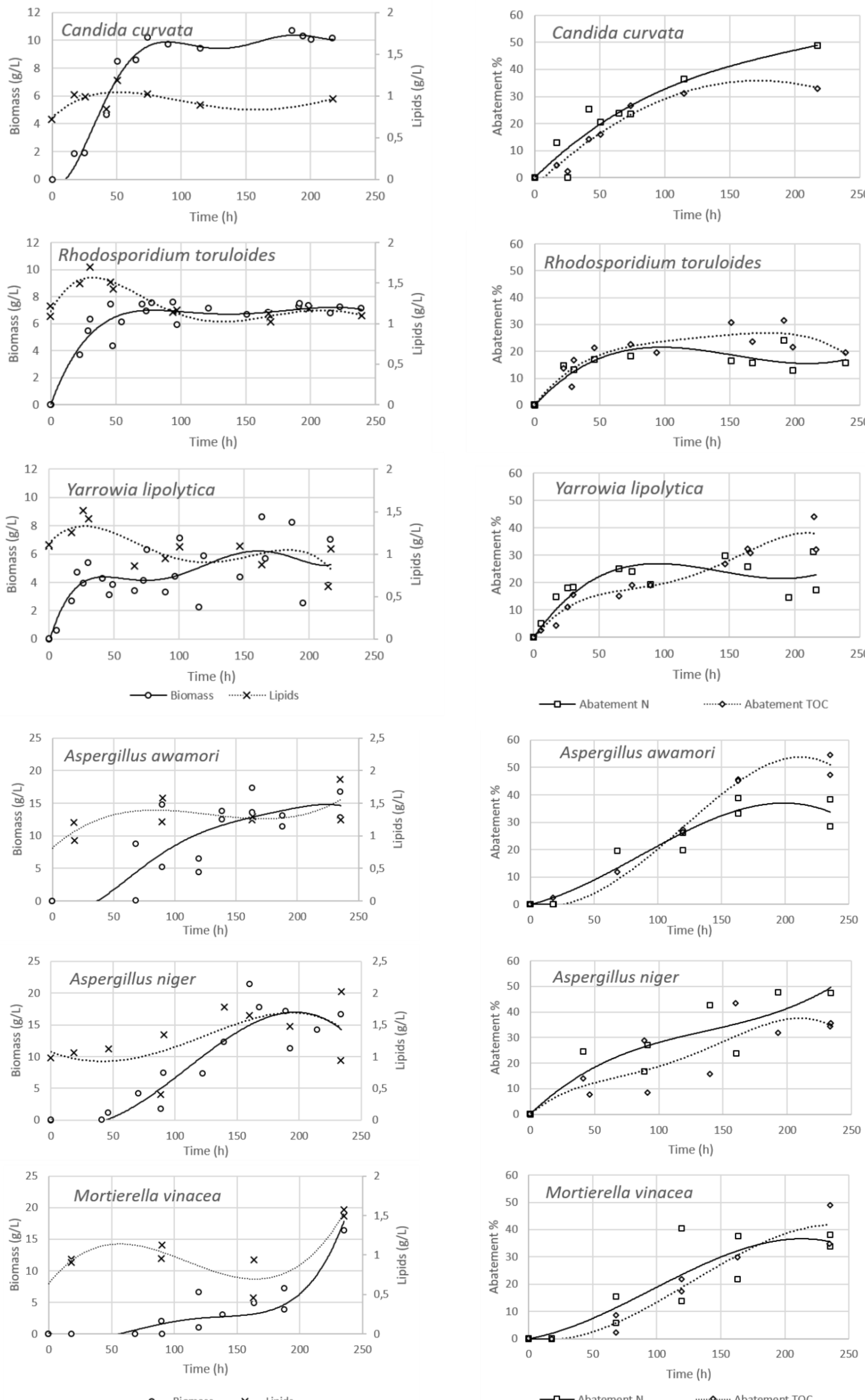

Figure 1. Kinetics of biomass and lipid production and carbon and nitrogen removal (% reduction) of six selected strains during growth on crude vinasse media.

Carbon and nitrogen uptake was also monitored during growth of the cells. This analysis globally showed a limited reduction of both elements comprised between 30% and 50% regardless the strain used in the experiment. The nutrients were therefore only partially metabolised by the microorganisms, illustrated by the low nitrogen consumption of 50% at best for *C. curvata* or *A. niger*. This phenomenon can be explained either by a nitrogen source in the DSW that cannot be metabolised by the cells, either by a limited concentration of another key and unidentified nutritive element.

However, for *Y. lipolytica*, the carbon uptake still occurred even after nitrogen consumption stopped. Given the fact that no additional biomass or lipid is produced in the meantime, this carbon uptake is probably used for another metabolic pathway yet to be determined.

3.3. Composition of the Lipids and Properties of the Biodiesel

The lipids produced by the selected 6 strains have been transesterified using acidic methanol to obtain fatty acid methyl ester (FAME). The FAMEs have been analysed by gas chromatography and their composition is presented in Figure 2 (see also Supplementary Materials), along with values from other feedstock from [54].

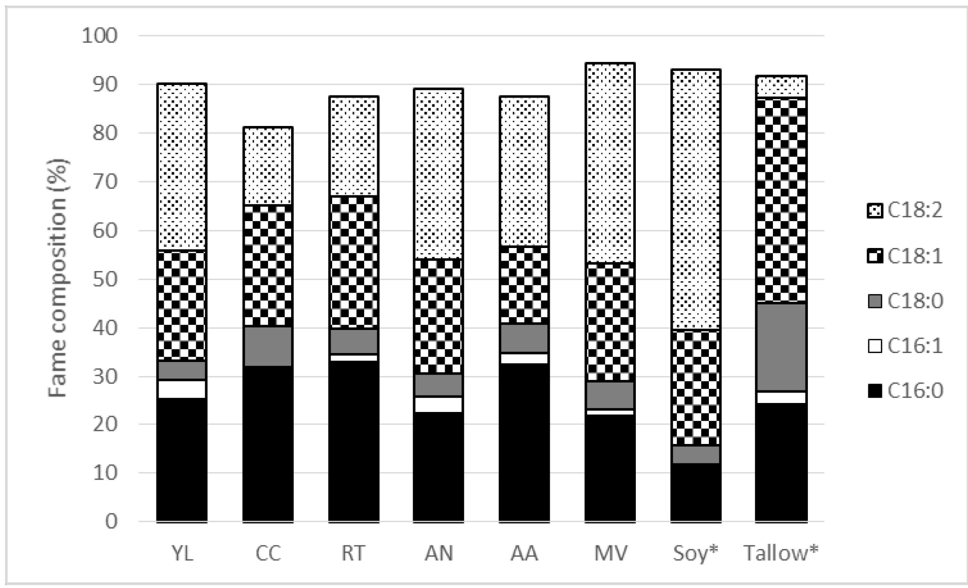

Figure 2. Fatty acid methyl ester (FAME) composition of the oils from six selected strains grown on pure DSW and comparison with other oil feedstock for biodiesel. YL: *Yarrowia lipolytica*; CC: *Candida curvata*; RT: *Rhodosporidium toruloides*; AN: *Aspergillus niger*; AA: *Aspergillus awamori*; MV: *Mortierella vinacea*. * Data from [54].

We found that oleic (C18:1, n-9), linoleic (C18:2, n-6), and palmitic (C16:0) acids represented more than 70% of total FAMEs, whereas palmitoleic (C16:1 n-7) and stearic (C18:0) acids accounted for less than 10% (Figure 2). These five molecules represented more than 80% of the total fatty acids esters. The composition of the microbial oil is therefore similar to oil from other feedstock used for biodiesel production. Soy oil have a high content of unsaturated fatty acid (C18:1, n-9 and C18:2, n-6) with a saturated/unsaturated (S/U) fatty acid ratio of 0.19. Tallow grease has a more important S/U ratio of 0.86. Most of the microbial oil S/U ratios fell within this range (between 0.41 and 0.78), except for *C. curvata* oil in which S/U ratio was higher at 0.98. Given that highly unsaturated oil (low S/U ratio) loses its storage capacity and that an excessively saturated one (high S/U ratio) loses its resistance to cold, it is foreseeable that single cell oils produced from DSW-grown microbial biomass presented good technical characteristics and should be suitable for biodiesel production.

Table 4 showed the properties of biodiesel calculated from the oil composition of the selected strains.

Table 4. Biodiesel properties of six selected strains determined from the composition of the oils.

	Cetane Number	Viscosity (mm^2/s)	Density (g/cm^3)	HHV (MJ/kg)	CFPP (°C)
A. niger	55.47 ± 2.74	3.12 ± 0.29	0.78 ± 0.06	35.75 ± 2.81	−1.88 ± 2.64
A. awamori	56.82 ± 2.51	3.08 ± 0.2	0.77 ± 0.05	35.08 ± 2.23	3.02 ± 0.25
M. vinacea	57.96 ± 1.51	3.33 ± 0.1	0.83 ± 0.02	37.84 ± 0.89	−0.58 ± 0.78
Y. lipolytica	56.49 ± 5.43	3.16 ± 0.42	0.79 ± 0.09	36.16 ± 4.29	−2.1 ± 1.21
C. curvata	56.25 ± 2.02	2.9 ± 0.12	0.71 ± 0.03	32.59 ± 1.28	7.04 ± 0.27
R. toruloides	58.89 ± 5.3	3.12 ± 0.04	0.76 ± 0.01	35.09 ± 0.55	2.06 ± 5.05
EN 14214	>51	3.5–5	0.85–0.9	49.65 *	<−15 [a]; <0 [b]

HHV: higher heating value; CFPP: cold filter plugging point; [a] limit for winter conditions in France; [b] limit for summer in France; * value for diesel, not defined by the European standard EN 14214.

The cetane number (CN) is a measure of a fuel's autoignition ability, i.e., a crucial parameter for compression-ignition engine. Viscosity of the fuel influenced the injection step of the oil in the combustion chamber. Higher viscosity led to poorer combustion of the fuel. Density is also a key property that affects engine performance. Because fuel pumps are volumetric, higher density fuel increases the energy input in the combustion chamber and affects the air/fuel ratio. The higher heating value (HHV) is a measure of the energy content of a fuel. This parameter is not included in the international standards but it gives information on the potential of over-consumption of the biodiesel fuel compared to diesel, as it is generally lower in biodiesel than in diesel. Cold filter plugging point (CFPP) represents the cold temperature behaviour of a fuel.

At low temperatures, saturated FAMEs form waxy solid precipitate and plug filter and fuel lines. Each country defines their standard based on seasonal temperatures. European standard EN 14214 defines the normalized values for each key parameters. Biodiesel specifications in the EU are defined by a CN of at least 51, a viscosity of 3.5 to 5 mm^2/s, a density of 0.86–0.90 g/cm^3, and a temperature range from 0 to −15 °C for summer or winter temperature in France. The higher heating value for diesel is set at 49.65 MJ/kg. All the tested strains gave a single cell oils with cetane number higher than required by the EN 14214. Viscosities and densities were slightly below specifications excepted for *C. curvata* which showed minimum values of 2.9 and 0.71 for viscosity and density respectively, and representing 82% of the specification value. Biodiesel contained about 30% less energy than diesel as indicated by low HHVs. *A. niger*, *M. vinacea* and *Y. lipolytica* produced FAMEs with cold flow properties suitable for summer conditions in France, with a CFPP value below 0 °C. Although calculation was done using 5 major FAMEs representing at least 80% of total FAMEs, the characteristics of the biodiesel determined here theoretically were close to EN 14214 specifications.

4. Conclusions

In the current economic context, it is becoming urgent to find new alternatives to produce low-cost biofuels. Oils derived from microorganisms are advantageous because they can develop continuously (continuous cultures or fed-batch) on low-cost resources such as agro-industrial wastes. By comparison with plants, cultivation of microorganisms is not subjected to the same climatic constraints (seasonality), nor is it affected by food and environmental issues related for example to overexploitation of the soil or deforestation resulting from the intensive cultivation of oleaginous plants [55].

The main purpose of this work was to select good microbial candidates for biodiesel production on DSW based on dried biomass and lipid production. Among the 28 strains screened, three yeast and three fungal strains were selected for further analysis. Our results demonstrated that they were able to grow and to produce promising amounts of lipids on pure DSW. Nutrients in the medium were only partially mobilised, suggesting a possible improvement of the production capacities. The biodiesel quality was similar to the one from traditional plant and animal feedstock and had properties close to EN 14214 standards, making them a promising source for potential biodiesel production from DSW.

However, it is still too early to try to estimate the efficiency of the cost of production of such a process because it has so far only been tested at laboratory scale. Moreover, the implementation of such an alternative process is limited by the context of low-cost fossil fuels that remain very competitive

despite the significant fluctuations of the market. To complete this work, the next step currently under investigation in the laboratory is therefore to optimize the biomass and lipid production yields (using fed batch mode and modified culture medium) in order to make this type of process more competitive and cost-effective.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4441/10/11/1623/s1, Table S1: Fatty acid composition (in %) of the best biomass and lipid producer strains, Figure S1: GC chromatogram of *A. awamori*, Figure S2: GC chromatogram of *A. niger*, Figure S3: GC chromatogram of *C. curvatus*, Figure S4: GC chromatogram of *M. vinacea*, Figure S5: GC chromatogram of *R. toruloides*, Figure S6: GC chromatogram of *Y. lipolytica*.

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