Biotechnology Reports 30 (2021) e00639

Contents lists available at ScienceDirect

Biotechnology Reports

journal homepage: www.elsevier.com/locate/btre

# The use of flow cytometry to assess *Rhodosporidium toruloides* NCYC 921 performance for lipid production using *Miscanthus* sp. hydrolysates

Joana Alves Martins, Teresa Lopes da Silva*, Susana Marques, Florbela Carvalheiro, Luísa B. Roseiro, Luís C. Duarte, Francisco Gírio

*Laboratório Nacional de Energia e Geologia, I.P., Unidade de Bioenergia e Biorrefinarias, Estrada do Paço do Lumiar 22, 1649-038, Lisboa, Portugal*

ARTICLE INFO

*Article history:*
Received 23 December 2020
Received in revised form 24 May 2021
Accepted 26 May 2021

*Keywords:*
*Rhodosporidium toruloides*
Lipids
*Miscanthus* sp.
Hydrolyzate
Capillary zone electrophoresis
Flow cytometry

ABSTRACT

The yeast *Rhodosporidium toruloides* NCYC 921 was used for lipid production, using *Miscanthus* biomass hydrolysate as carbon source. The hydrolysate was obtained by enzymatic hydrolysis of *Miscanthus* biomass (at high solids loading) previously subjected to a hydrothermal pre-treatment. Afterwards *R. toruloides* was grown on *Miscanthus* sp. hydrolysate (MH), undiluted and diluted, at the ratios of 1:4 (20 % v/v), 1:2 (33.3 % v/v) and 3:1 (75 % v/v). The best yeast performance was observed for MH 1:2 medium dilution, reaching the maximal biomass concentration of 6.3 g/L, the lipid content of 30.67 % w/w dry cell weight and the lipid concentration of 1.64 g/L. Flow cytometry demonstrated that *R. toruloides* cell membrane was massively damaged when the yeast was grown on undiluted MH, due to the presence of phenolic compounds; however, when the yeast was grown on diluted MH 1:2 and 1:4, the proportion of intact cells has increased during the yeast cultivation.


## 1. Introduction

Oleaginous microorganisms can accumulate intracellular lipids above 20 % of their dry weight [1] which can be further extracted and converted into biofuels, namely biodiesel and/or biojet fuel, (e.g., Hydrotreated Esters of Fatty Acids (HEFA) [2]. However, advanced biofuels from oleaginous microbes, including microalgae, still require further developments to become fully economically sustainable, since its production cost is still not competitive when compared to low price fossil fuels or even first generation biofuels [3].

A possible approach to improve process sustainability consists of using low cost culture media, such as those obtained from lignocellulosic industrial by-products and residues. However, these materials exhibit a recalcitrant structure, based on three main polymers - cellulose, hemicellulose and lignin - and a remaining smaller part of non-structural components that may include pectin, protein, extractives and ash. The structural carbohydrates, which typically account for more than half of the total dry weight of the lignocellulosic biomass, can be used as sugars source for microbial lipid heterotrophic production. However, the release of its cellulose and hemicellulose derived monosaccharides (glucose and mainly xylose, respectively) requires the application of a pre-treatment step followed by an enzymatic hydrolysis step [4]. This pre-treatment step aims to separate lignin and hemicellulose fractions, reducing cellulose crystallinity, increasing the porosity of lignocellulose, and minimizing chemical destruction of fermentable sugars required for the subsequent fermentation step [5]. However, this step inevitably generates some fermentation inhibitor compounds derived both from biomass composition (i.e. acetic acid and phenolic compounds) or from sugars degradation reactions (e. g., organic acids - formic and levulinic acids, and furan derivates - 5-hydroxymethylfurfural (HMF) and furfural). As a result, culture media based on lignocellulosic sugars will also contain these potential inhibitory compounds that may affect cell viability and metabolic activity, decreasing the lipid production [6].

The inhibitory effect of these compounds on microbial cells physiology and metabolism has been extensively reported, mainly for bioethanol production processes, as well as detoxification methods available to alleviate its effect [5,7–9]. A few works have described the negative effect of these inhibitory compounds on yeast growth and lipid production [10,11] but, as far as the authors know, their effect on yeast cell physiological status has not been reported before.

Several lignocellulosic materials, including wheat straw [12], corn cobs [13], corn stover [14], wood residues [15] and *Miscanthus* sp. [12], have been used in media formulation to grow oleaginous yeasts. *Miscanthus*, also called silvergrass, is a highly productive C4 perennial grass that has been considered as a feedstock for

* Corresponding author.
*E-mail address:* teresa.lopessilva@lneg.pt (T. Lopes da Silva).

https://doi.org/10.1016/j.btre.2021.e00639

bioenergy applications. The grass cultivation has been studied and applied in Europe and USA, namely to produce heat, electricity and bioethanol [16].

Mast et al. [12] reported the use of non-detoxified hydrolysates obtained from *Miscanthus* sp. biomass as carbon source to produce lipids by *Rhodotorula glutinis*, attaining a low lipid production (~ 7 % w/w DCW of the yeast biomass, providing 0.93 g/L) which was attributed to the rapid sugar exhaustion after 96 h of growth. However, these authors did not evaluate the stress physiological response of the yeast cells exposed to such conditions, which might have contributed for the low lipid production.

The red yeast *Rhodosporidium toruloides* NCYC 921 is an oleaginous microorganism that also produces carotenoids of high commercial interest, which are used as natural food colorants and feed additive in aquaculture [17,18]. In the present work, this yeast was grown on *Miscanthus* sp. hydrolysate (MH) as carbon source to produce intracellular lipids. As a preliminary study, the yeast was grown on semi-defined medium containing increasing initial glucose concentrations, in order to evaluate the yeast tolerance to sugars. For the first time, the growth of *R. toruloides* NCYC 921 using MH as culture medium was monitored by flow cytometry, in order to evaluate the impact of MH on the yeast membrane integrity. Such information, obtained near real time, allows the fast process optimization to obtain the highest lipid productivities.

## 2. Materials and methods

Fig. 1 schematically depicts the process studied in the present work for production of lipids by *R. toruloides* NCYC 921 using *Miscanthus* sp. biomass.

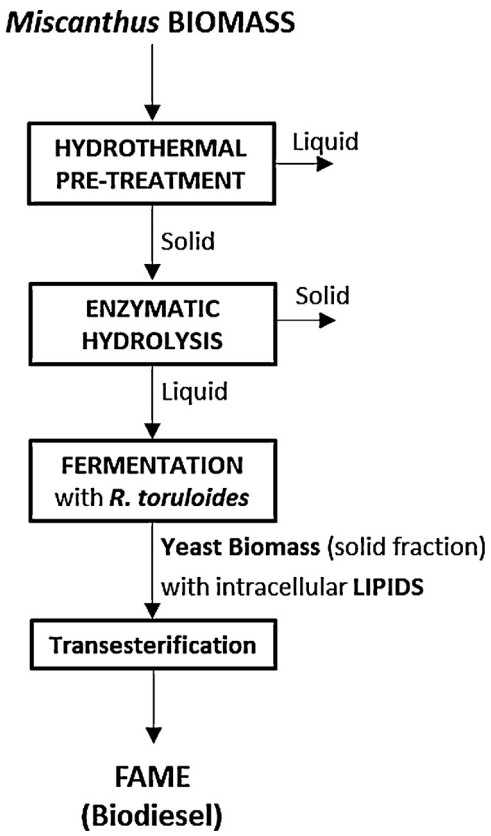


**Fig. 1.** Process for production of lipids (further converted into FAME) by *Rhodosporidium toruloides* NCYC 921 using *Miscanthus* biomass.

### 2.1. Feedstock

*Mischantus* sp. biomass was purchased from Comgoed (NL) by TU Delft and distributed by TNO within the consortium of Brisk 2 project. The solid was supplied as pellets with moisture content of 12 % (w/w) and was stored in plastic containers at room temperature, prior to use.

### 2.2. Preparation of Miscanthus sp. hydrolysate

#### 2.2.1. Hydrothermal pre-treatment

In order to release sugar monomers from *Miscanthus* sp. biomass, this was subject to hydrothermal processing (autohydrolysis) as pre-treatment, prior to enzymatic hydrolysis.

The hydrothermal pre-treatment was carried out in a 2-L stainless steel reactor (Parr Instruments Company, Moline, IL, USA) by applying a liquid:solid ratio of 8:1 (g water/g feedstock), at 190 °C for 15 min. After pre-treatment, the solid and liquid fractions were separated by hydraulic pressing. The solid fraction was then washed with water (two volumes of water used in the autohydrolysis) at room temperature and directly used for the enzymatic hydrolysis step. The pre-treated solid was weighted and the moisture was determined using an infrared balance (AMB). A sample of the solid was dried (45 °C, 48 h) and chemically characterised, as described in 2.4.1 section.

#### 2.2.2. Enzymatic hydrolysis

*Miscanthus* sp. hydrolysate (MH) was obtained after 72-h enzymatic saccharification of the hydrothermally pre-treated solid suspended in 0.05 M sodium citrate buffer, pH 5.0, at an initial 150 g/L solids concentration (oven-dried basis), by applying Cellic® C-Tec2 (Novozymes, Denmark) at a cellulase dosage of 15 FPU/g solids. The *Miscanthus* sp. suspension was sterilised by autoclaving (20 min, 121 °C) and further incubated with the enzyme solution previously sterilised (through a membrane filter of 0.22-μm pore size) at 50 °C in an orbital shaker (150 rpm). After 72 h, the resulting hydrolysate was centrifuged (6000×g, 15 min, 10 °C) under sterile conditions to remove the unreacted solids and then appropriately stored (4 °C) until use. Hydrolysates were analysed for the content of sugars, acetic acid and phenolic compounds as described in Section 2.4.

The conversion yield for enzymatic hydrolysis of cellulose into glucose - corresponding to the ratio of total glucan equivalent hydrolysed to total potential cellulose available in the initial pre-treated substrate - was determined according to the following equation:

$$\eta C = \frac{M_g \; x \; f_{hg}}{M_i \; x \; C_i} x \; 100$$

Where $\eta_C$ (%) is the enzymatic hydrolysis yield for cellulose; $M_g$ – mass of glucose (g) in MH; $M_i$ - mass (oven-dried basis) of pre-treated *Miscanthus* used in the enzymatic hydrolysis (g); $C_i$ – cellulose content (mass fraction) in the pre-treated *Miscanthus*; $f_{hg}$ - correction factor for hydration on converting glucose into cellulose (0.9).

Similarly, the conversion yield for enzymatic hydrolysis of xylan into xylose was determined according to the following equation:

$$\eta X = \frac{M_x \; x \; f_{hx}}{M_i \; x \; X_i} \; x \; 100$$

Where $\eta_X$ (%) is the enzymatic hydrolysis yield for xylan; $M_X$ – mass of xylose (g) in MH; *Mi* - mass (oven-dried basis) of pre-treated *Miscanthus* used in the enzymatic hydrolysis (g); $X_i$ -xylan content (mass fraction) in the pre-treated *Miscanthus*; $f_{hx}$ – correction factor for hydration on converting xylose into xylan (0.88).

### 2.3. Lipid production

#### 2.3.1. Microbial strain and maintenance

The yeast *R. toruloides* NCYC 921 (former *R. glutinis* NRRLY-1091) was purchased from the National Collection of Yeast Cultures (Norwich, UK). The strain was stored on slants of Malt Extract Agar, at 4 °C.

#### 2.3.2. Culture conditions

For inocula preparation, *R. toruloides* yeast cells from two slants grown for 72 h at 30 °C were transferred to baffled 1 L-Erlenmeyers containing different growth media, as described below. After 1 day-growth (exponential growth phase) the inoculum (5 %, v/v) was transferred to 1 L-baffled shake flasks containing 150 mL of the corresponding sterile culture medium.

To study the yeast tolerance to increasing sugar concentrations, 150 mL of nitrogen-excess semi-defined culture medium, as described by Li et al. [19], was used: $(NH4)_2SO_4$ 12.0 g/L, $KH_2PO_4$ 1.0 g/L, $MgSO_4{\cdot}7H_2O$ 1.5 g/L, yeast extract 0.5/L; glucose was added as carbon source, at concentrations ranging from 10 to 200 g/L. The cultures were incubated at 30 °C, 150 rpm, for 100 h, in order to ensure that all cultures attained the stationary phase.

For the other assays, a nitrogen-limiting semi-defined culture medium, as described by Yoon and Rhee [20], was used: $CaCl_2{\cdot}2H_2O$ 0.134 g/L, $MgSO_4$ 0.73 g/L; $Na_2HPO_4$ 2 g/L, $KH_2PO_4$ 7 g/L, yeast extract 0.5 g/L, $(NH4)_2SO_4$ 1 g/L; undiluted or diluted MH was added to the culture medium as carbon source, at various dilution ratios [1 part of MH: 2 parts of culture medium (33.3 % v/v); 1 part of MH:4 parts of culture medium (20 % v/v); and 3 parts of MH :1 part of culture medium (75 % v/v)]. For the MH assay, with the best results in terms of lipid production, a further control assay was performed using the semi-defined medium supplemented with the same glucose and xylose concentrations present in the corresponding MH dilution, for comparison. In this case, sugars were separately sterilised and mixed after cooling with the other components of the culture medium. For all the experiments, the media pH was adjusted to 4.0 before inoculation [17]. The cultures were incubated at 30 °C, 150 rpm, for 72 h.

Along the cultivation, 1-mL samples were collected for analysis of yeast biomass weight and sugars consumption (as described in 2.4.2 and 2.4.3).

### 2.4. Analytical methods

#### 2.4.1. Chemical characterisation of lignocellulosic biomass

Feedstock and pre-treated biomass were gravimetrically analysed for water (by oven drying at 105 °C to constant weight) and ash (by applying NREL/TP-510-42622 protocol [21]) contents. Polysaccharides (hemicellulose and cellulose) and lignin contents were assayed by means of a quantitative hydrolysis with sulphuric acid in two stages (the first step with 72 % (w/w) acid at 30 °C for 1 h; and the second with 4 % (w/w) acid for 1 h at 121 °C) based on NREL/TP-510-42618 protocol [22]. The quantification of the liberated monosaccharides and acetic acid was carried out by HPLC (as described in 2.4.3). The acid insoluble residue was considered as Klason lignin, after correction for the acid insoluble ash. The acid-soluble lignin was determined in the corresponding filtrate by UV spectroscopy at 320 nm (NREL/TP-510-42618 protocol [22]).

#### 2.4.2. Yeast biomass quantification

For the yeast tolerance assay, the yeast biomass was measured turbidimetrically by optical density at 600 nm in a spectrophotometer Spectronic GENESYS 20 (USA), calibrated against dry cell weight (as determined at 100 °C to constant weight, over 18 h). For the assays using MH, 1 mL of samples taken from the cultures was centrifuged (at 13 000 rpm, in a bench centrifuge (Haeraeus Sepatech Biofuge 15, Germany) for 5 min in pre-weighted 1.5 mL Eppendorfs. The supernatant was used for sugar HPLC analysis (as described below) and the biomass pellet was dried overnight in an oven at 100 °C. For each MH dilution, the dry weight of the corresponding culture medium supplemented with MH without cells was determined using the same protocol, and this fraction was further deducted from the whole pellet weight to obtain the final cell mass weight.

#### 2.4.3. HPLC analysis

Monosaccharides (glucose, xylose, arabinose), acetic acid and furans (HMF and furfural) were analysed by HPLC using an Aminex HPX-87H column (Bio-Rad, USA) in an Agilent HPLC (Germany), equipped with a diode array detector (DAD) and a refractive index detector (RI) under the conditions described before [23].

#### 2.4.4. Lipid quantification

*R. toruloides* NCYC 921 lipids were extracted according to the protocol described by Freitas et al. (2014) with modifications [24]. The yeast biomass collected by broth centrifugation was freeze-dried (Heto PowerDry LL3000 Freeze Dryer, Thermo Scientific, USA, coupled with a vacuum pump from Vacuubrand, Germany). Freeze-dried biomass (approximately 100 mg) was transferred to a vial under nitrogen atmosphere and transmethylated, at 80 °C for 1 h, with 2 mL of a methanol/acetyl chloride mixture (95:5 v/v) and adding 0.2 mL of heptadecanoic acid (17:0) (5 mg/mL petroleum ether, boiling point 80−100 °C) as internal standard. Afterwards, the vial contents were cooled, diluted with 1 mL water and the lipids were extracted with 2 mL n-heptane. The organic phase was separated from the aqueous phase, dried ($Na_2SO_4$) and placed in a vial adequate for gas chromatography analysis.

The methyl esters were then analysed on a gas–liquid chromatograph (Bruker Scion 436-GC, Germany) equipped with a flame ionization detector. Separation was carried out on a 0.32 mm ×30 m fused silica capillary column (film 0.32 mm) Supelcowax 10 (Supelco, Bellafonte, Palo Alto, CA, USA) with helium as a carrier gas, at a flow rate of 3.5 mL/min. The column temperature was programmed at an initial temperature of 200 °C for 8 min, then increased at 4 °C/min to 240 °C and hold there for 16 min. Injector and detector temperatures were 250 °C and 280 °C, respectively, and the split ratio was 1:50 for 5 min and then 1:10 for the remaining period. The column pressure was 13.5 psi. Peak identification and response factor calculation were carried out using known standards (GLC 459 and GLC 463, Nu-Chek-Prep, USA). Each sample was prepared in duplicate and injected twice.

#### 2.4.5. Flow cytometry

Flow Cytometry analysis was performed in a BD FACSCalibur™ cytometer (Becton, Dickinson and Company, USA). For the analysis in the flow cytometer, 3 mL samples were collected from culture broth under sterile conditions and stored in Falcon tubes. These were subsequently subjected to ultrasounds treatment for 10 s, to disintegrate cellular aggregates. All samples were diluted in PBS buffer. In this study, Propidium iodide (PI) and thiazole orange (TO) were used, in combination, for distinguishing cells with intact membrane from cells with permeabilized/compromised membrane. TO enters all cells and binds to DNA, discriminating cells from debris, while PI cannot cross an intact cytoplasmic membrane. PI was used at a concentration of 2 mg/L and was detected in the FL3 channel. TO was used at a concentration of 0.1 mg/L, which was detected in the FL1 channel. For control purposes, unstained samples were recorded to take into account the autofluorescence of the cells. At least 2 replicates were made for each different dye used. The obtained cytograms were processed

and imaged using the Flowing Software version 2.5.0 (Perttu Terho).

*2.4.6. Total phenolic content of Miscanthus hydrolysate*

Total phenolics were determined using the Folin–Ciocalteu colorimetric method according to the procedure described by Roseiro et al. [25], adapted to a microplate format using spectrophotometric detection and microtiter 96-well plates. Gallic acid was used as standard and several concentrations were prepared for a calibration curve. Aliquots of 200 μL were placed in each microplate well and after 40 min-incubation at room temperature, absorbance was measured at 725 nm on a microplate reader (Multiscan GO, ThermoFischer Sc.). The whole procedure was done in triplicate and results expressed as g of gallic acid equivalent (GAE)/L sample.

*2.4.7. Phenolic profile by capillary zone electrophoresis*

Phenolic profile was obtained by capillary zone electrophoresis (CZE) using an Agilent Technologies CE system (Waldbronn, Germany) equipped with a diode array detector (DAD), as described in Roseiro et al. [25]. Electropherograms (e-grams) were recorded at 200 and 280 nm, and phenolic compounds were identified with data from authentic standards run under the same conditions and stored in library.

## 3. Results and discussion

### 3.1. Preliminary assays for sugar inhibition

In order to study the effect of high sugar concentrations on *R. toruloides* NCYC 921 growth and lipid production, batch experiments were conducted in 1 LErlenmeyers with initial glucose concentrations ranging from 10 to 200 g/L. The kinetic parameters obtained for this preliminary set of experiments are presented in Table 1. The specific growth rate (μ) slightly varied for the assays with initial glucose concentrations from 10 to 130 g/L, attaining ~ 0.20 $h^{-1}$. However, for assays at initial glucose concentrations above 130 g/L it markedly decreased, reaching 0.12 $h^{-1}$ for the assay at 200 g/L glucose (almost 60 % of the specific growth rate observed for the assays at initial glucose concentrations of 10−130 g/L). These results demonstrate that the yeast growth was inhibited for initial glucose concentrations above 130 g/L. The maximum biomass concentration slightly varied between 2.24 and 2.92 g/L, for all the assays.

In addition, *R. toruloides* NCYC 921 lipid production was maximal for the assay using 90 g/L of glucose (11.1 % w/w DCW of the yeast biomass).

Similar results were reported by Li et al. [19] using the yeast *R. toruloides* Y4 grown in medium with the same composition containing increasing initial glucose concentrations from 10 to 400 g/L. These authors reported that the yeast grew on media containing glucose at concentrations up to 150 g/L. The maximum biomass concentration was observed at 40 g/L glucose, decreasing for glucose concentrations higher than 40 g/L. Similar trend has been observed for the effect of increasing glucose concentrations on *R. toruloides* Y4 lipid production.

These results demonstrate that *R. toruloides* NCYC 921 growth is inhibited for glucose concentrations higher than 130 g/L, and lipid synthesis is maximal for 90 g/L of glucose concentration.

### 3.2. Preparation of Miscanthus hydrolysate (MH)

Table 2 shows the composition of the *Miscanthus* biomass used in this work (asreceived and after pre-treatment). This feedstock contains 71.4 % (w/w) of total polysaccharides, from which 60 % is cellulose, as estimated from the glucan content. The glucan and xylan contents of this *Miscanthus* fall, respectively, within the ranges of 28.7–46.4 % and 19.6–27.1 % respectively, encountered in the compositional analysis of 80 *Miscanthus* genotypes reported by Zhang et al. [26]. The values for polysaccharides contents favourably compare with other lignocellulosic feedstocks, such as different types of straws and wood residues [23,27].

The hydrothermal pre-treatment of *Miscanthus* mainly affected hemicellulose components and 80.6 %, 100 % and 85.6 % of the original xylan, arabinan and acetyl groups, respectively, were solubilised. In contrast to hemicellulose, cellulose was completely recovered in the solid phase, remaining potentially available for conversion into glucose (to be further assimilated by the yeast) by the subsequent enzymatic hydrolysis, as desirable. A similar behaviour was found for the hydrothermal pre-treatment of other lignocellulosic materials, e.g. wheat straw, rice straw or olive tree pruning [28], but with a lower glucan recovery in some reports. The hydrothermal pre-treatment also promotes some effect in Klason lignin as 10.4 % of the original lignin was solubilised. Although there are some cases wherein no apparent lignin solubilisation occurs due to the autohydrolysis processing, these solubilisation levels have often been reported for the pre-treatment of biomass using these processes [28]. Indeed, high enzymatic yields can be achieved by some pre-treatments that increase cellulose surface without removal of hemicellulose or lignin [29]. However, several authors [30,31] have demonstrated a significant effect on the hydrolysis yield for cellulose in lignocellulosic biomass by removing hemicellulose and lignin in the pre-treatment step.

Cellic® CTec2, a commercial cellulase cocktail also exhibiting high levels of xylanase activity, was used to carry out the enzymatic hydrolysis of the polysaccharides present in pre-treated *Miscanthus* (cellulose and xylan) into their monosaccharides (glucose and xylose) that would be metabolised by the yeast to produce lipids. Once the preliminary assays have shown that lipid synthesis by *R. toruloides* NCYC 921 is maximal for 90 g/L of glucose, in order to get a sugars concentration as close as possible to this titre, enzymatic hydrolysis was carried out by resuspending pre-treated *Miscanthus* at a high solids concentration of 150 g/L. The composition of the hydrolysate obtained after 72 h-reaction at 50 °C, together with the corresponding saccharification yields, is presented in Table 3.

**Table 1**
*R. toruloides* specific growth rate and lipid content (t = 100 h) when grown on culture semi-defined medium containing initial glucose concentrations 10-200 g/L.

| Initial glucose concentration (g/L) | μ ($h^{-1}$) | Lipids (% w/w DCW) |
|---|---|---|
| 10 | 0.21 ± 0.04 | 7.4 ± 0.2 |
| 30 | 0.23 ± 0.05 | 6.9 ± 0.6 |
| 50 | 0.22 ± 0.00 | 6.0 ± 0.6 |
| 70 | 0.18 ± 0.00 | 7.6 ± 0.5 |
| 90 | 0.20 ± 0.01 | 11.1 ± 0.6 |
| 130 | 0.18 ± 0.03 | 5.9 ± 0.3 |
| 160 | 0.11 ± 0.01 | 5.1 ± 0.0 |
| 200 | 0.12 ± 0.00 | 5.6 ± 0.3 |

**Table 2**
Chemical composition (major constituents) of *Miscanthus* – as received and pretreated.

| Constituent | Composition (g/100 $_{g\ oven\text{-}dried}$) | |
|---|---|---|
| | As-received | Pre-treated |
| Glucan | 42.4 | 62.8 |
| Xylan | 21.5 | 5.8 |
| Arabinan | 6.7 | 0.0 |
| Acetyl | 4.0 | 0.8 |
| Klason lignin | 23.7 | 29.5 |
| Acid soluble lignin | 1.7 | 1.1 |

**Table 3**
Yields of glucan and xylan conversion into glucose and xylose ($\eta_{cellulose}$, $\eta_{xylan}$), respectively and corresponding final sugars concentrations for the enzymatic hydrolysis of pre-treated *Miscanthus* biomass using Cellic® CTec2.

| $\eta_{celluose}$ (%) | $\eta_{xylan}$ (%) | [Glucose] (g/L) | [Xylose] (g/L) | [Acetate] (g/L) | [Total sugars] (g/L) |
|---|---|---|---|---|---|
| 53.5 | 72.7 | 58.8 ± 0.1 | 7.1 ± 0.1 | 1.1 ± 0.1 | 65.9 ± 0.1 |

The saccharification yield observed for the enzymatic hydrolysis of cellulose in the pre-treated biomass is inferior to 55 %, meaning that the hydrolysis was limited as compared to the extension of hydrolysis achieved in previously reported studies with *Miscanthus*. Despite hydrothermal pre-treatment having solubilised xylan in *Miscanthus* to a high extent (80 %), leading to an increase in cellulose (glucan) and lignin contents, its effectiveness is relatively limited taking into account the yield achieved in the subsequent enzymatic hydrolysis step.

For instance, Liu et al. [32] achieved a glucose yield of 85 % for enzymatic hydrolysis of 20 g/L of *Miscanthus* x *giganteus* pre-treated by a two-step alkaline process at 150 °C that promoted a 89 %-delignification yield along with 60 % of hemicellulose solubilisation and 80 % of cellulose recovery. When *Miscanthus* x *giganteus* was subjected to dilute oxalic acid pre-treatment under conditions of very high severity, Scordia et al. [33] attained a glucose yield of 91 % for enzymatic hydrolysis at a biomass concentration of 100 g/L. However, high levels of acetic acid (9.0 g/L), furfural (9.43 g/L), hydroxymethilfurfural (0.75 g/L) and phenolics have been detected in the liquid fraction obtained in the pre-treatment, that could also be present in the pre-treated biomass, hampering the subsequent fermentation of the hydrolysate. Murnen et al. [34] reported a glucose yield of 96 % for enzymatic hydrolysis of 10-g/L glucan concentration in AFEX-treated *Miscanthus*.

It can thus be concluded that *Miscanthus* sp. is a recalcitrant energy crop, requiring harsh pre-treatment conditions to deconstruct, and probably higher lignin solubilisation together with hemicellulose removal will be advantageous.

However, no matter the differences in the pre-treatment applied, all these authors have subjected *Miscanthus* sp. to enzymatic hydrolysis under much lower solids concentrations (20−100 g/L in comparison to the 150-g/L concentration applied in the present study) thus, getting very low sugars concentrations in the hydrolysates that would be insufficient to achieve a reasonable lipid titre. Although the glucose titre achieved in the hydrolysate of the present study is lower than the 90-g/L level providing maximal lipids production by *R. toruloides* NCYC 921, the authors opted for sacrificing the yield in favour of a higher sugar concentration that would favour the yeast lipid production. Indeed, it is extensively reported that the enzymatic hydrolysis is very limited due to rheology constrains when dealing with high concentrations of solid feedstock that significantly increases the viscosity of the reaction mixture [35].

Thereby, the work proceeded by using this hydrolysate obtained from 150 g/L of pre-treated *Miscanthus* sp., for culturing *R. toruloides* NCYC 921. Indeed, the total sugars concentration of 65.9 g/L (corresponding to 58.8 g/L of glucose and 7.1 g/L of xylose) present in MH potentially allows its use as culture medium for *R. toruloides* NCYC 921, bearing in mind the preliminary substrate inhibition experiments demonstrating that this yeast strain is able to grow in media containing up to 130 g/L of sugars.

### 3.3. Yeast growth on MH based media

The yeast did not grow on undiluted MH, which suggests that *R. toruloides* cells were inhibited by potential toxic compounds present in this hydrolysate, since the sugars concentration present in MH (65.9 g/L) was lower than the critical concentration that inhibited the yeast growth, previously determined (>130 g/L). This result might be unexpected, since the pre-treated biomass has been extensively washed prior to the enzymatic hydrolysis step (as described in Section 2.2.1), that would eliminate any potential inhibitory compounds produced in the pre-treatment step.

Since furan derivatives were not found in undiluted MH and the acetic acid concentration is very low (∼1.12 g/L), the existence of other possible inhibitory compounds, such as phenolic compounds, was investigated by capillary zone electrophoresis (CZE).

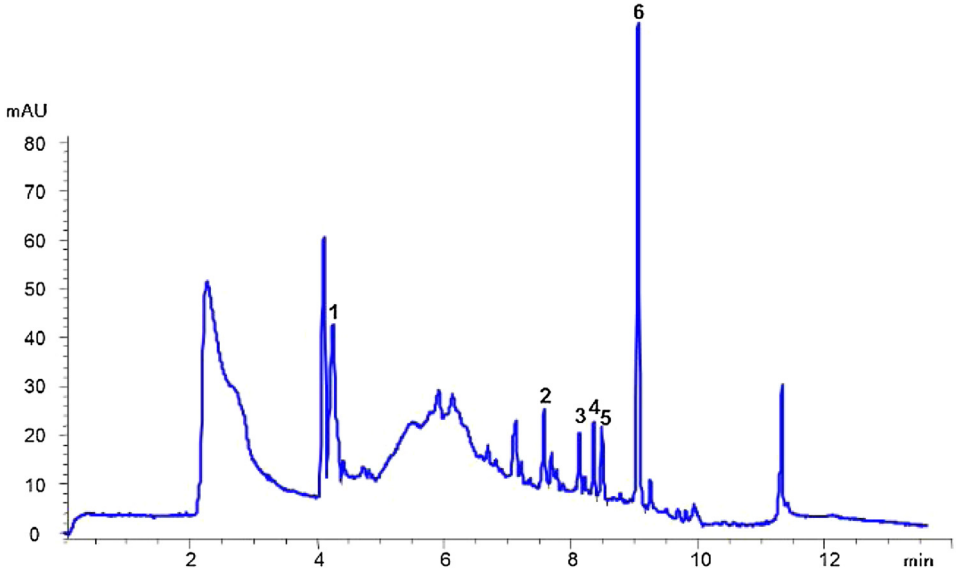


**Fig. 2.** Phenolic compounds profile for pure HM by capillary electrophoresis, and the similarity percentages between the identified compounds and standards. 3HBAc – 3-hidroxibenzoic acid; 4HBAld – 4- hydroxibenzaldeid; V – vanilin; CA – Cumaric Acid; VA – Vanili Acid; V - Vanilin.

In fact, the phenolic profile obtained for MH by CZE revealed a complex matrix (Fig. 2). However, with the available standards, it was only possible to identify the presence of vanillic acid, vanillin, p-coumaric acid and 4-hydroxybenzaldehyde, which are phenolic compounds typically present not only in *Miscanthus* sp. bark [36,37] but also in its extracts obtained by organosolv fractionation [38]. These compounds are well-known by their biological activities, in particular antifungal activity [39].

Vanillin and other hydroxybenzaldehyde derivatives are also common phenolic compounds resulting from lignocellulosic pretreatments [40] and thus the presence of these compounds in MH should be expectable. As reported by Zhao et al. [10], vanillin inhibits *R. toruloides* AS 2.1389 growth when present in concentrations equal or higher than 2.0 g/L. Nevertheless, the total concentration of phenolic compounds assayed by the Folin-Ciocalteu method for undiluted MH was only 0.4 g/L, which is lower than the 2.0 g/L inhibiting concentration for vanillin as reported by Zhao et al. [10]. According to these authors, when formic acid, acetic acid, furfural and vanillin were tested, separately, in the culture medium, their concentrations should be lower than 2, 5, 0.5 and 1.5 g/L, respectively, to allow the yeast growth. However, the authors have verified that the synergistic effects of these compounds, even present at low concentrations in the growth medium, could dramatically decrease their critical inhibitory concentrations, leading to significant inhibition of the yeast growth and lipid production. Thereby, it is possible that the synergistic effect of all phenolics, together with the presence of acetic acid, may have contributed for the complete inhibition of *R. toruloides* growth on undiluted MH.

In addition, phenolic compounds have the ability to bind to proteins promoting inactivation by decreasing their solubility and/or forming enzyme-inhibitor complexes [41]. Thus, this inhibitor effect can also have contributed for the low yields observed for the previous enzymatic hydrolysis step.

Therefore, in order to dilute the potential inhibitory compounds present in MH, assays on semi-defined culture medium supplemented with MH at different dilutions were carried out.

Fig. 3 shows *R. toruloides* profiles obtained for the yeast cultivation on semi-defined medium supplemented with MH, at various dilutions (1 vol of MH: 2 volumes of semi-defined medium (1:2 or 33.3 % v/v); 1 vol of MH:4 volumes of semi-defined medium (1:4 or 20 % v/v) ; 3 volumes of MH: 1 vol of semi-defined medium (3:1 or 75 % v/v).

Both assays with MH at 1:2 and 1:4 dilutions allowed the yeast growth (Fig. 3 a)), which indicates that the MH should be diluted (at least at a ratio of 1:2) prior to its use as carbon source for the yeast growth. The highest biomass concentration (6.3 g/L, Table 4) was observed for the assay with MH 1:2, at t = 47.5 h; in the assay with MH 1:4, biomass reached 3.9 g/L at t = 25.6 h (Table 4). No yeast growth was observed in the assay with the highest MH concentration (MH 3:1), which supports the raised hypothesis that the hydrolysate contains inhibitory compounds that negatively affect the cell growth.

The variation of the culture broth pH during the assays is depicted in Fig. 3 b). In both assays with MH 1:2 and 1:4, pH decreased until t = 24 h, increasing during the stationary phase, until the end of the fermentation, reaching final pH values > 5.0. When *R. toruloides* grows on glucose, pH of culture broth always decreases throughout the cultivation, since acidic metabolites (mainly acetic acid) are typically produced [42]. Concerning the assay with MH 3:1, no change in pH was observed throughout the yeast cultivation, demonstrating that no cell metabolism was exhibited during this assay.

Sugars consumption profiles are displayed in Fig. 3 c) and d). In both MH 1:2 and 1:4 assays, glucose and xylose were completely exhausted at t = 48 h, although glucose was faster consumed than xylose, revealing that the yeast has a preference for glucose, as expected. There was no sugar consumption for the 3:1 assay, also confirming the absence of cell metabolism.

Total lipids content produced by *R. toruloides* at the end of cultivation is shown in Fig. 3 e). The highest lipid content was observed for the assay with MH 1:2 (30.67 % w/w DCW of the yeast biomass), followed by the assays with MH 1:4 (20.08 % w/w DCW of the yeast biomass) and MH 3:1 (3.20 % w/w DCW of the yeast biomass). Therefore, beyond cell growth, MH also inhibited the yeast lipid synthesis.

These results demonstrate 1:2 as the best MH dilution for yeast growth and lipid synthesis.

Fig. 3 f), g), h) and i) compares the yeast growth and lipid production profiles for the assay with MH 1:2 and the semi-defined culture medium control (containing reagent-grade glucose and xylose as carbon sources, at the same concentration of MH 1:2 medium). The comparison of these profiles highlights the benefits of using MH as carbon source for *R. toruloides* growth and lipid synthesis, as alternative to the use of commercial-grade glucose and xylose monosaccharides. At t = 48 h, the MH 1:2 assay showed an increase in biomass concentration of 53.6 % as compared to the control experiment (Fig. 3 a) and f)). The profiles for broth pH (Fig. 3 g) depicted the differences between the yeast growth on MH and the control assay. The increase observed in pH for the MH 1:2 assay might be explained by the activity of the enzyme phenylalanine/tyrosine ammonia lyase present in *R. toruloides* (Uniprot) (https://www.uniprot.org/uniprot/P11544), which catalyses the conversion of the amino acids L-phenylalanine and L-tyrosine, precursors of lignin synthesis to trans-cinnamate and p-coumaric acid, respectively, releasing $NH4^+$ in the medium with the subsequent pH increase [43].

The sugar consumption is depicted in Fig. 3 h). In MH 1:2 assay, both glucose and xylose were exhausted at t = 48 h. Indeed, in this assay, glucose was consumed faster than in the control assay. Moreover, in the latter, glucose was not completely exhausted, and no xylose consumption was observed till t = 72 h.

The yeast lipid content assessed in the biomass collected at the end of the yeast cultivations (t = 72 h) was 38 % higher in the MH 1:2 assay than in the control assay (30.67 % w/w DCW and 22.21 % DCW, respectively) (Fig. 3 i). This increase in lipid production could be due to the presence of additional nutrients (besides sugars) in the diluted 1:2 MH hydrolysate, such as phenolic compounds. These can be assimilated by the yeast after (or concomitantly) with the monosaccharides consumption, favouring the lipid production during the stationary phase. Actually, Nogué et al. [44] reported that *R. toruloides* was also able to grow on four aromatic compounds present in corn stover hydrolysate, as a sole carbon source, suggesting its use as a strain for simultaneous sugar and lignin conversion. Yaegashi et al. [45] have also reported the ability of *R. toruloides* to metabolize lignin-related aromatic phenolic compounds other than p-coumaric acid, also present in lignocellulosic hydrolysates, such as p-hydroxybenzoic acid (4-HBA). In fact, the phenolic profile obtained for MH indicates the presence of 3hydroxybenzoic acid (3-HBA) with 96 % matching (data not shown) as 4-HBA standard was not available. Therefore, *R. toruloides* NCYC 921 cells might also have consumed potential nutrients such as phenolic compounds, or others, present in diluted MH as an additional carbon source. This probably explains the higher yeast lipid content observed for MH 1:2 assay, as compared to the control assay.

Table 4 displays the kinetic parameters for the yeast growth and lipid production on MH 1:2 and 1:4 assays. It also compares to the control assay at the same glucose and xylose concentrations of MH 1:2, and the results obtained by Mast et al. [12]. These authors described the growth and lipid production by an oleaginous yeast (*Rhodotorula glutinis* CBS 20) carried out in a 5L-bioreactor, using

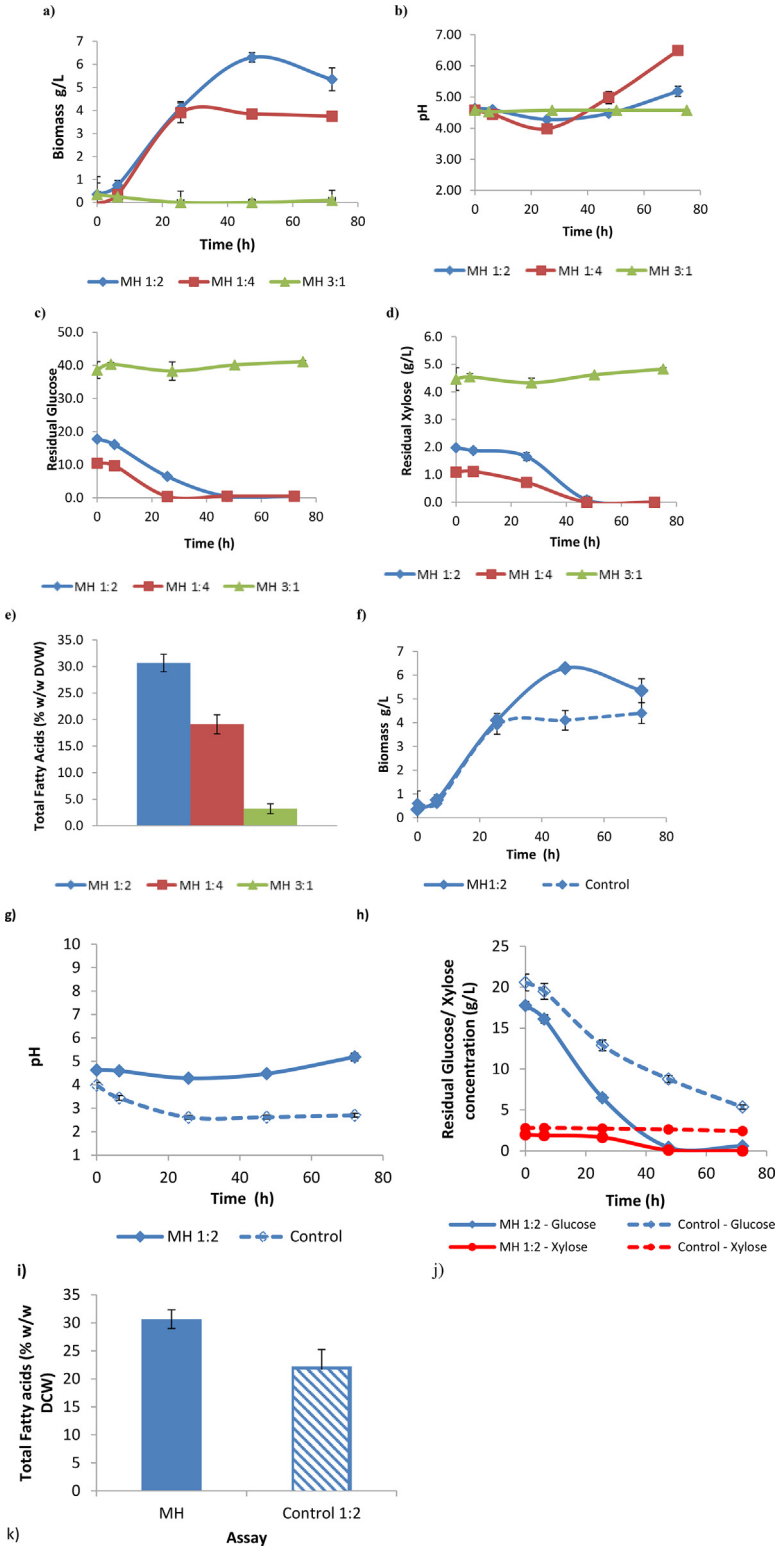


**Fig. 3.** *R. toruloides* growth on pure MH, and diluted MH 1:2, 1:4 and 3:1.
a) Biomass profiles.
b) Medium pH profiles.
c) Residual glucose.
d) Residual xylose.
e) Total fatty acids at t = 72 h.
f) Biomass profiles for MH 1:2 assay and respective control.
g) Medium pH profiles for MH 1:2 assay and respective control.
h) Residual glucose and xylose for MH 1:2 assay and respective control.
i) Total Fatty acids for MH 1:2 assay and respective control.

**Table 4**
Kinetic parameters concerning *R. toruloides* growth and lipid production on MH 1:4, 1:2 and corresponding control, and 3:1. Comparison with the results obtained by Mast et al. (2014).

| Assay | Cultivation system | $X_{max}$ (g/L) | $r_{x\ max}$ (g/L/h) | Lipid content (% w/w DCW) | Lipid concentration (g/L) | Lipid productivity (g/L/h) | $Y_{L/S}$ g lipids/ g total sugars |
|---|---|---|---|---|---|---|---|
| MH 1:4 | Batch (1L-Erlenmeyers | 3.9 ± 0.4 (t = 25.6 h) | 0.15 ± 0.02 | 20.08 ± 2.40 (t = 72 h) | 0.75 ± 0.09 (t = 72 h) | 0.010 ± 0.001 (t = 72 h) | 0.07 |
| MH 1:2 | Batch (1L-Erlenmeyers | **6.3 ± 0.2** (t = 47.5 h) | 0.13 ± 0.00 | **30.67 ± 1.67 (t = 72 h)** | **1.64** ± 0.09 **(t = 72 h)** | **0.023** ± 0.001 **(t = 72 h)** | **0.09** |
| Control of assay MH 1:2 | Batch (1L-Erlenmeyers | 4.2 ± 0.3 (t = 72 h) | 0.06 ± 0.00 | 22.21 ± 3.01 (t = 72 h) | 0.98 ± 0.17 (t = 72 h) | 0.014 ± 0.002 (t = 72 h) | 0.06 |
| MH 3:1 | Batch (1L-Erlenmeyers | 0 | 0 | 0 | 0 | 0 | 0 |
| Mast et al. 2014 | 5L-bioreactor | 11.7 (t = 168 h) | 0.07 | ~9.00 (t = 96 h) ~7.00 (t = 168 h) | 0.93 (t = 96) 0.83 (t = 168 h) | 0.006 (t = 96 h) 0.005 (t = 168 h) | 0.05 |

$r_x$ – Maximum biomass productivity: calcucated as the ratio between themaximum biomass concentration and the cultivation time at which the maximum biomass was observed.
Lipid concentration: calculated as the ratio between the lipid content and the biomass concentration at t = 72 h.
Lipid productivity: calcucated as the ratio between the lipid concentration and the cultivation time (72 h).
Lipid Yield $Y_{L/S}$ : Lipid yield- calculated as the ratio between the lipid concentration and the total sugars consumed at t = 72 h.

MH supplemented with a nutrient solution promoting a dilution ratio of 5.2:1.0 (84 % v/v), providing a total sugars concentration of 15.4 g/L. Such a bioreactor configuration should presumably favour the yeast growth and lipid synthesis, since neither nutrient limiting conditions (especially oxygen) nor pH variations are usually present in bioreactor cultivations, due to the efficient mixing conditions and pH control, but are often present in shake flasks cultures. However, it can be seen that lipid content, concentration, productivity and yield were higher for the assays conducted in the present work, using 1-L Erlenmeyers. This increase is particularly notorious for the MH 1:2 assay, attaining 30.67 % w/w DCW, 1.64 g/L, 0.023 g/(L h and 0.09 g lipids/ g total sugars, respectively for lipid content, concentration, productivity and yield. Therefore, the lower lipid production (0.93 g/L) reported by Mast et al. [12] is probably due to the much lower total sugar content of the MH used as carbon source (16 g/L), or to the presence of acetic acid, furfural and HMF reported by the authors, or even to the presence of phenolic compounds, since they have used a culture medium containing MH almost undiluted (supplemented with only 8% v/v).

### 3.4. Fatty acid methyl esters (FAME) composition

Table 5 displays the FAME composition of *R. toruloides* biomass collected at the end of the experiments (72 h). The dominant FAME are oleic (18:1ω9), linoleic (18:2ω6) and palmitic acids (16:0), comprising more than 85 % of total fatty acids (TFA) in all cases. The main differences between FAME composition of *R. toruloides* biomass grown on MH and the control assay concerns the proportion of 18:1ω9. In MH assays, oleic acid percentage comprises more than 57 % of TFA; whereas in the control assay this proportion was only 46.43 % of TFA. In addition, the linoleic and linolenic acids (18:2ω6 and 18:3ω3) proportions were higher for the control assay. As a result, the proportion of monounsaturated methyl esters (MUME) was higher for MH assays than for the control, which should confer advantages in terms of stability for the biodiesel obtained from *R. toruloides* FAME. In fact, according to the report of Ridque [46], the optimal biodiesel would be only composed of MUME. An ideal biodiesel composition should have fewer polyunsaturated methyl esters (PUME) and saturated methyl esters (SME). High levels of PUME reduce the biodiesel oxidative stability and increase nitrogen oxide emissions, which do not suit diesel engines. On the contrary, biodiesel composed of SME would exhibit good oxidative stability, but poor fuel properties at low temperatures, which is a disadvantage during winter [47].

**Table 5**
*R. toruloides* fatty acids composition for MH 1:4, MH 1:2 and control (with the same sugar concentration of MH 1:2) assays.

| | MH | | Control |
|---|---|---|---|
| Fatty acid | MH 1:4 | MH 1:2 | |
| 12:0 | 0.05 (± 0.04) | 0.04 (± 0.01) | 0.02 (± 0.00) |
| 14:0 | 0.73 (± 0.09) | 0.86 (± 0.02) | 0.71 (± 0.01) |
| 16:0 | 14.11 (± 0.27) | 17.98 (± 0.13) | 13.14 (± 0.28) |
| 16:1ω9 | 0.76 (± 0.02) | 0.61 (± 0.02) | 0.51 (± 0.01) |
| 18:0 | 4.01 (± 0.15) | 5.20 (± 0.09) | 4.42 (± 0.08) |
| 18:1ω9 | **58.97 (± 0.52)** | **56.66 (± 0.31)** | **46.53 (± 0.65)** |
| 18:2ω6 | **16.99 (± 0.29)** | **14.70 (± 0.24)** | **27.87 (± 0.41)** |
| 18:3ω3 | **1.81 (± 0.04)** | **1.87 (± 0.06)** | **4.81 (± 0.06)** |
| 20:0 | 0.15 (± 0.00) | 0.21 (± 0.01) | 0.20 (± 0.00) |
| 20:1ω11 | 0.29 (± 0.01) | 0.20 (± 0.00) | 0.24 (± 0.00) |
| 22:0 | 0.52 (± 0.02) | 0.58 (± 0.17) | 0.47 (± 0.00) |
| 24:0 | 1.62 (± 0.08) | 1.09 (± 0.08) | 1.07 (± 1.51) |
| SME(%) | 21.19 (± 0.29) | 25.97 (± 0.16) | 20.03 (± 1.13) |
| MUME (%) | 60.01(± 0.50) | 57.47 (± 0.33) | 47.28 (± 0.67) |
| PUME (%) | 18.80 (± 0.32) | 16.57 (± 0.30) | 32.68 (± 0.47) |

SME – Saturated methyl esters.
MUNE – monounsaturated methyl esters.
PUME – Polyunsaturated methyl esters.

In addition, according to the European Standard EN 14214, the percentages of linolenic methyl ester (18:3) and PUME with more than three double bonds should be lower than 12 % and 1 % (w/w), respectively. In this work, the percentage of linolenic acid was always lower than 5%, and there is no PUME with more than three double bonds in *R. toruloides* FAME composition. Therefore, *R. toruloides* FAME composition presents an advantage over the vegetable oils, the current feedstock used to produce biodiesel, since many of these vegetable oils contain a high level of linolenic and palmitic acids [47] requiring the addition of additives to the oil-based biodiesel to enhance its utilization, with the subsequent process cost increase. In addition, microbial oils do not compete with food applications and, therefore, there are no sustainability concerns for the use of this type of oils for biofuels.

### 3.5. Yeast physiological response

#### 3.5.1. Flow cytometric controls

To demonstrate the feasibility of assessing the membrane integrity of *R. toruloides* NCYC 921 by uisng flow cytometry coupled with the mixture of TO and PI fluorochromes, a serie of controls were preliminary assessed, using yeast cells at different physiological states. In these controls, metabolically active exponentially growing cells, and heat-treated injured cells were used. As can be

seen in Fig. 4-I, 91.98 % of *R. toruloides* cells were stained with TO, but not with PI, meaning that those cells had intact membrane (subpopulation A); only 8.01 % of the cells were stained with TO and PI, so these cells had permeabilized membrane (subpopulation B). Fig. 4-II shows the dot-plot/cytogram for *R. toruloides* heat-treated cell stained with the TO /PI mixture. Almost 100 % of the cells were stained with TO and PI in Fig. 4.1 II, subpopulation B), confirming the cytoplasmic membrane damage that resulted from the heat treatment. This test confirmed that the mixture of TO/PI dyes, in association with flow cytometry, can successfully differentiate subpopulations of *R. toruloides* cells with intact membrane from cells subpopulations with damaged membrane (Fig. 5).

*3.5.2. Yeast cultivations*

Fig. 4 a)–l) shows the flow cytometric data for *R. toruloides* grown on MH diluted at the ratios of 1:2, 1:4 and 3:1.

The proportion of cells with permeabilised membrane (subpopulation B) determined during the stationary phase increased during the assay using the most concentrated MH (MH 3:1), attaining 98.78 % at the end of the cultivation (Fig. 4 a)–c), revealing that most of the yeast cells were exposed to such harsh and adverse conditions that damaged their membrane, possibly due to the presence of inhibitor compounds at higher concentrations than in the assays with other dilutions of MH. In fact, it is known that phenolic compounds act on biological membranes, causing loss of cell membrane integrity [5]. This data supports the results displayed in Fig. 4a)–e) which showed that the yeast cells did not grow when using MH 1:3.

For MH assays at 1:2 and 1:4 dilutions, the opposite was observed. A gradual increase in the proportion of intact cells (subpopulation A) throughout the yeast cultivation was observed from the exponential phase to the late exponential phase (Fig. 4 d)–f); Fig. 4g)–i)), and the proportion of injured cells was lower than 1.5 % at the end of the cultivation (Fig. 4 f) and i)). These results suggest that the phenolic compounds present in the diluted MH did not affect the yeast cell membrane, because they were present in lower concentrations than in the assay with MH 3:1. In addition, the cells possibly consumed these compounds, after glucose and xylose exhaustion, which allowed their metabolic activity maintenance, including membrane integrity and lipid synthesis, even during the late stationary phase (Fig. 4 e)). Such results are in agreement with the previous CZE results, which revealed the presence of consumable phenolic compounds by yeasts, according to cited works [44,45].

The control assay showed a similar *R. toruloides* subpopulation pattern during the cultivation development (Fig. 4 j)–l)) comparing to the assays with MH (dot plots). The proportion of subpopulation B slightly decreased from the exponential to the stationary phase, attaining 13.90 % at the end of the cultivation (Fig. 4 l)), which is higher than the proportion of subpopulation B observed at the end of the assays with MH (< 1.5 %). This indicates that the yeast cells experienced harsher conditions during the control assay, possibly due to the broth pH drop observed during this assay, since the optimal pH for *R. toruloides* growth is 4.0 [42].

## 4. Conclusions

*R. toruloides* has effectively grown on semi-defined culture medium supplemented with diluted MH (1:2) attaining a lipid content of ~31 % w/w DCW. The different yeast stress responses when grown on MH at different dilutions, monitored by flow cytometry, indicated that high concentrations of MH negatively affected the yeast growth, and damaged the cell membrane, probably resulting from the presence of potential inhibitor compounds in the MH. However, when diluted MH was used as carbon source, some compounds present in MH (such as phenolics), now at lower concentrations, could possibilby be uptaken by the cells as nutrients, allowing to maintain the yeast membrane integrity, as well as cell growth and lipid production.

*R. toruloides* MUME proportion (> 50 % TFA) obtained in the yeast biomass collected from the assays with MH represents an additional advantage over vegetable oils used as fedstock for biodiesel.

Additionally, the approach here reported can also be used to co-produce lipids and carotenoids from *R. toruloides* NCYC 921, since this strain also produces carotenoids with commercial interest. Such approach could boost the economics of the whole process, bearing in mind the high market value of the carotenoids produced by this yeast strain.

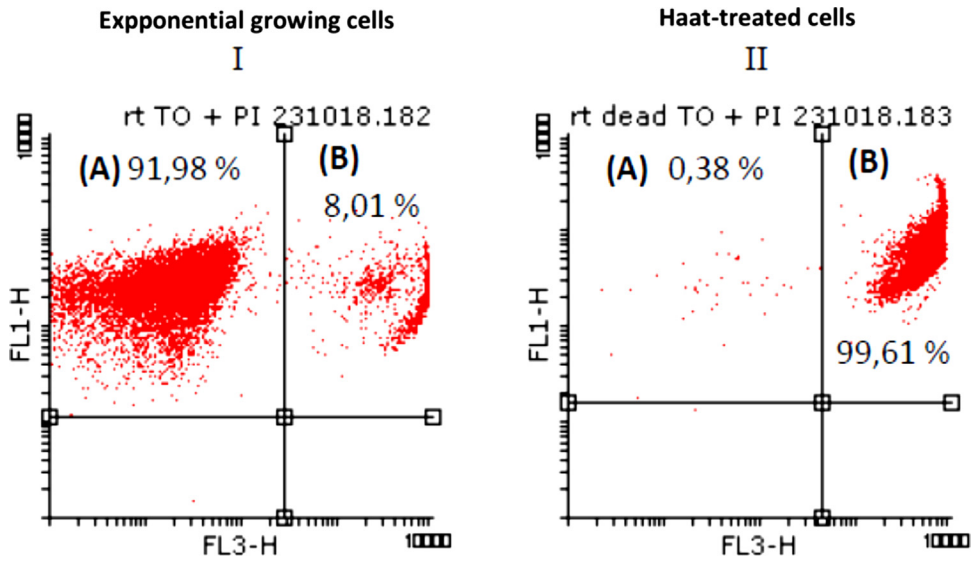


**Fig. 4.** *R. toruloides* flow cytometric dot plots controls.
I – exponential growing cells; II – heat-treated cells.
Subpopulation A – cells with intact membrane.
Subpopulation B – cells with permeabilised (injured) membrane.

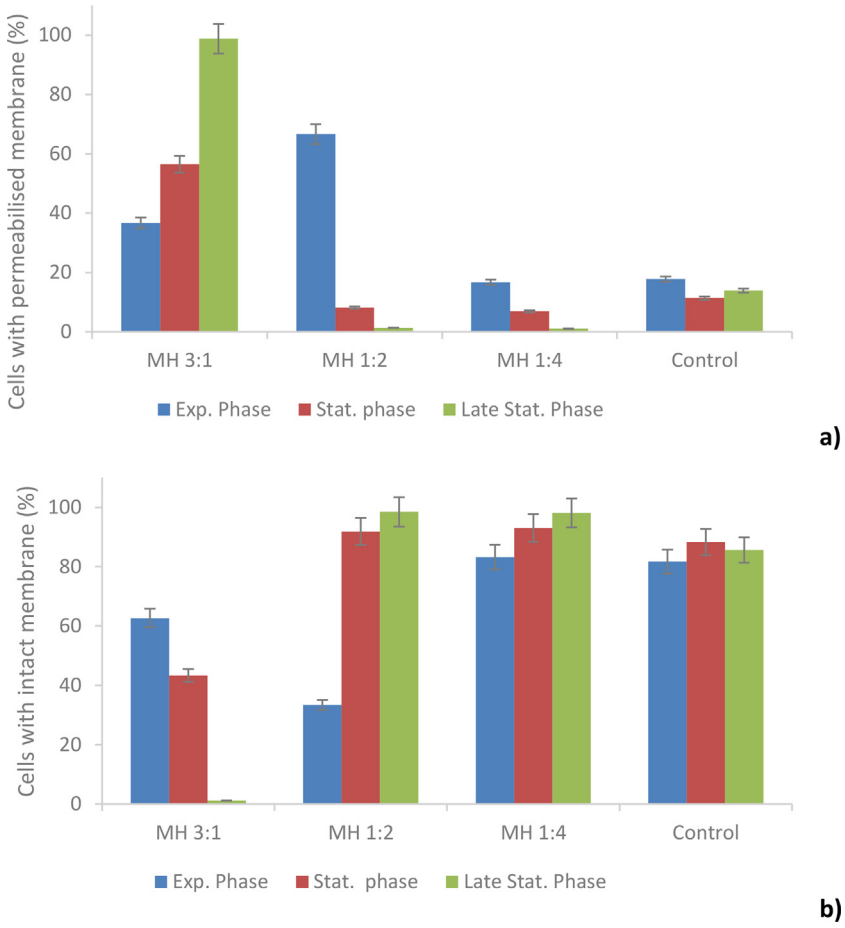


**Fig. 5.** a) *R. toruloides* cells with permeabilised membrane (%). b) R. *toruloides* cells with intact membrane (%); Error bars show the standard deviation (n = 2).

Since nutritional limitations often occur in shake flaskes cultivations, limiting the process efficiency, *R. toruloides* cultivations on diluted MH (1:2) developed in bench and semi-pilot bioreactors are now in progress, in order to improve biomass and lipid productivities.

## Contributions

JAM carried out the laboratorial work. TLS supervised the experimental work concerning the yeast cultivations, lipid quantification and flow cytometry analysis; SM supervised the experimental work concerning the enzymatic hydrolysis; FC and LCD supervised the experimental work concerning the pre-treatment; LR supervised the experimental work concerning the capillary zone electrophoresis; TLS drafted the manuscript with contributions provided by all the co-authors.

## Declaration of Competing Interest

The authors report no declarations of interest.

## Acknowledgements

The present work has received funding from Horizon 2020's Research and Innovation Programme under grant agreement number 731101 (BRISK2 project: Biofuels Research Infrastructure for Sharing Knowledge).

This work was carried out at the Biomass and Bioenergy Research Infrastructure (BBRI-LISBOA-01-0145-FEDER-022059), which is supported by Operational Programme for Competitiveness and Internationalization (PORTUGAL 2020), by Lisbon Portugal Regional Operational Programme (Lisboa 2020) and by North Portugal Regional Operational Programme (Norte 2020) under the Portugal 2020 Partnership Agreement, through the European Regional Development Fund (ERDF).

Cellic® CTec2 was kindly provided by Novozymes A/S (Denmark).

The authors thank Dr. Margarida Monteiro (MM) and Céu Penedo (CP) for laboratory support and strain maintenance.

## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.btre.2021.e00639.

## References

[1] I. Gientka, M. Kieliszek, K. Jermacz, S. Blazejak, Identification and characterization of oleaginous yeast isolated from kefir and its ability to accumulate intracellular fats in deproteinated potato wastewater with different carbon sources, Biomed Res. Int. (2017) 6061042.
[2] X. Zhao, X. Sun, D. Cui, Production of biojet fuels from biomass, in: M. Rai, A. Ingle (Eds.), Sustainable Bioenergy, Elsevier, Amsterdam, The Netherlands, 2019, pp. 127–165.
[3] T. Braunwald, W.T. French, W. Claupein, S. Graeff-Hönninger, Economic assessment of microbial biodiesel production using heterotrophic yeasts, Int. J. Green Energy 13 (2016) 274–282.
[4] F.M. Gírio, C. Fonseca, F. Carvalheiro, L.C. Duarte, S. Marques, R. Bogel-Łukasik, Hemicelluloses for fuel ethanol: a review, Bioresour. Technol. 101 (2010) 4775–4800.
[5] J.R.M. Almeida, T. Modig, A. Petersson, B. Hähn-Hägerdal, G. Lidén, M.F. Gorwa-Grauslund, Increased tolerance and conversion of inhibitors in lignocellulosic

hydrolysates by *Saccharomyces cerevisae*, J. Chem. Technol. Biotechnol. 82 (2007) 340–349.
[6] T. Lopes da Silva, A. Reis, in: A.S.Ozkan Atta-ur-Rahman (Ed.), Low-Cost Feedstocks for Biofuels and High Value Added Products Production: Using Multi-Parameter Flow Cytometry as a Tool to Enhance the Process Efficiency in Recent Advances in Analytical Techniques, 32019, pp. 40–60 Bentham ebooks.
[7] L. Jönsson, B. Alriksson, N. Nilvebrant, Bioconversion of lignocellulosic: inhibitors and detoxification, Biotechnol. Fuels 6 (2013) 16.
[8] F. Carvalheiro, L.C. Duarte, S. Lopes, J.C. Parajó, H. Pereira, F.M. Gírio, Evaluation of detoxification methods on brewery's spent grain hydrolysate for xylitol production by *Debaryomyces hansenii* CCMI 941, Proc. Biochem. 40 (2005) 1215–1223.
[9] L.C. Duarte, F. Carvalheiro, I. Neves, F.M. Gírio, The effects of aliphatic acids, furfural and phenolic compounds in *Debaryomyces hansenii* CCMI 941, Appl. Biochem. Biotechnol. 121-124 (2005) 413–425.
[10] X. Zhao, F. Peng, W. Du, C. Liu, D. Liu, Effects of some inhibitors on the growth and lipid accumulation of oleaginous yeast *R. toruloides* and preparation of biodiesel by enzymatic transesterification of the lipid, Bioproc. Biosyst. Eng. 35 (2012) 993–1004.
[11] S. Rahman, P. Arbter, M. Popovi, R. Bajpai, R. Subramanuiam, Microbial lipid production from lignocellulosic hydrolyzates: effect of carbohydrate mixtures and acid-hydrolysis byproducts on cell growth and lipid production by *Lipomyces starkeyi*, J. Chem. Technol. Biotechnol. 92 (2017) 1980–1989.
[12] B. Mast, N. Zöhrens, F. Schmidl, R. Hernandez, W. French, N. Merkt, W. Claupein, Graeff-Hönnonger, Lipid production for microbial biodiesel by the oleaginous yeast *Rhodotorula glutinis* using hydrolysates of what straw and *Miscanthus* as carbon sources, Waste Biomass Valor. 5 (2014) 955–962.
[13] C. Huang, X. Chen, L. Xiong, X. Chen, Oil prodution by the yeast *Trichosporon dermatis* cultured in enzymatic hydrolysates of corncobs, Bioresour. Technol. 110 (2012) 711–714.
[14] I.R. Setipu, J. Fernandez, L. Sousa, V. Balan, K.L. Boundy-Mills, Identification of oleaginous yeast strains able to accumulate high intracellular lipids when cultivated in alkaline pretreated corn stover, Appl. Microbiol. Biotechnol. 98 (2014) 7635–7657.
[15] G. Tasseli, S. Filippucci, E. Borsella, S.D.' Antonio, M. Gelosia, G. Cavalaglio, B. Turchetti, C. Sannino, A. Onofri, S. Mastrolitti, I. De Bari, F. Cotana, P. Buzzini, Yeast lipids from cardoon stalks, stranded driftwood and olive tree pruning residues as possibel extra source of oils for producing biofuels amd biochemicals, Biotechnol. Biofuels 147 (2018) 11.
[16] E. Anderson, R. Arundale, M. Maughan, A. Oladeinde, A. Wycislo, T. Voigt, Growth and agronomy of Miscanthus x giganteus for biomass production, Biofuels 2 (2011) 71–87.
[17] C. Dias, S. Sousa, J. Caldeira, A. Reis, T. Lopes da Silva, New dual-stage pH control fed-batch cultivation strategy for the improvement of lipids and carotenoids production by the red yeast *R. toruloides* NCYC 921, Bioresour. Technol. 189 (2015) 309–318.
[18] P. Passarinho, B. Oliveira, C. Dias, M. Teles, A. Reis, T. Lopes da Silva, Sequential carotenoids extraction and biodiesel production from *Rhodosporidium toruloides* NCYC 921 biomass, Waste Biomass Valor. 11 (2020) 2075–2086.
[19] Y. Li, Z.K. Zhao, F. Bai, High-density cultivation of oleaginous yeast *Rhodosporidium toruloides* Y4 in fed-batch culture, Enzyme Microbiol. Technol. 41 (2007) 312–317.
[20] S.H. Yoon, J.S. Rhee, Lipid from yeast fermentation: effects of cultural conditions on lipid production and its characteristics of *Rhodotorula glutinis*, JAOCS 60 (1983) 1281–1286.
[21] A. Sluiter, B. Hames, R. Ruiz, C. Scarlata, J. Sluiter, D. Templeton, Determination of Ash in Biomass, NREL/TP-510-42622, National Renewable Energy Laboratory, USA, 2008.
[22] A. Sluiter, B. Hames, R. Ruiz, C. Scarlata, J. Sluiter, D. Templeton, D. Crocker, Determination of Structural Carbohydrates and Lignin in Biomass, National Renewable Energy Laboratory NREL/TP-510-42618, USA, 2012.
[23] H. P.Moniz, T. Pereira, Quilhó, F. Carvalheiro, Characterization and hydrothermal processing of corn straw towards the selective fractionation of hemicelluloses, Ind. Crops Prod. 50 (2013) 145–153.
[24] C. Freitas, B. Nobre, L. Gouveia, J.C. Roseiro, A. Reis, T. Lopes da Silva, New at-line flow cytometric protocols for determining carotenoid content and cell viability during *Rhodosporidium toruloides* NCYC 921 batch growth, Process Biochem. 49 (2014) 554–562.
[25] L.B. Roseiro, C.S. Tavares, J.C. Roseiro, A.P. Rauter, Antioxidants from aqueous detoction of carob pods biomass (*Ceretonia siliqua L.*): optimisation using response surface methodology and phenolic profile by capillary electrophoresis, Ind. Crops Prod. 44 (2013) 119–126.
[26] T. Zhang, C.E. Wyman, K. Jakob, B. Yang, Rapid selection and identification of Miscanthus genotypes with enhanced glucan and xylan yields from hydrothermal pretreatment followed by enzymatic hydrolysis, Biotechnol. Biofuels 5 (2012) 56.
[27] T. Silva-Fernandes, L.C. Duarte, F. Carvalheiro, M.C. Loureiro-Dias, C.F. Fonseca, F.G. Girio, Hydrothermal pretreatment of several lignocellulosic mixtures containing wheat straw and two hardwood residues available in Southern, Europe Biores. Technol. 183 (2015) 213–220.
[28] F. Carvalheiro, L.C. Duarte, F.M. Gírio, P. Moniz, in: S.I. Mussatto (Ed.), Hydrothermal/liquid Hot Water Pretreatment (Autohydrolysis): a Multi-Purpose Process for Biomass Upgrading in Biomass Fractionation Technologies for a Lignocellulosic Feedstock Based Biorefinery, 1st edition, Elsevier, 2016, pp. 315–347.
[29] J.A. Rollin, Z. Zhu, N. Sathitsuksanoh, Y.H.P. Zhang, Increasing cellulose accessibility is more important than removing lignin: a comparison of cellulose solvent-based lignocellulose fractionation and soaking in aqueous ammonia, Biotechnol. Bioeng. 108 (2011) 22–30.
[30] X. Zhao, L. Zhang, D. Liu, Biomass recalcitrance. Part I: the chemical and physical structures affecting the enzymatic hydrolysis of lignocelluloses, Biofuels Bioprod. Bioref. 6 (2012) 465–482.
[31] L. Feng, L. Qin, Z. Liu, C. Dong, B.-Z. Li, Y.-J. Yuan, Combined severity during pretreatment chemical and temperature on the saccharification of wheat straw using acids and alkalis of differing strength, BioResources 9 (2014) 24–38.
[32] Z. Liu, S. Padmanabhan, K. Cheng, H. Xie, A. Gokhale, W. Afzal, H. Na, M. Pauly, A. Bell, J. Prausnitz, Two-step delignification of *Miscanthus* to enhance enzymatic hydrolysis: aqueous ammonia followed by sodium hydroxide and oxidants, Energy Fuels 28 (2014) 542–548.
[33] D. Scordia, S.L. Cosentino, T.W. Jeffries, Effectiveness of dilute oxalic acid pretreatment of *Miscanthus* x *giganteus* biomass for ethanol production, Biomass Bioenergy 59 (2013) 540–548.
[34] H.K. Murnen, V. Balan, S.P.S. Chundawat, B. Bals, L.C. Sousa, B.E. Dale, Optimization of Ammonia Fiber expansion (AFEX) pretreatment and enzymatic hydrolysis of *Miscanthus* x *giganteus* to fermentable sugars, Biotechnol. Prog. 23 (2007) 846–850.
[35] S. Marques, F.M. Gírio, J.A.L. Santos, J.C. Roseiro, Pulsed fed-batch strategy towards intensified process for lactic acid production using recycled paper sludge, Biomass Convers. Bio. 7 (2017) 127–137.
[36] J.J. Villaverde, R.M.A. Domingues, C.S.R. Freire, A.J.D. Silvestre, C.P. Neto, P. Ligero, A. Vega, *Miscanthus x giganteus* Extractives: a source of valuable phenolic compounds and sterols, J. Agric. Food Chem. 57 (2009) 3626–3631.
[37] N. Brosse, A. Dufour, X.Q. MengSun, A. Ragauskas, *Miscanthus*: a fast-growing crop for biofuels and chemicals bioproduction, Biofuels Bioprod. Bioref. 6 (2012) 580–598.
[38] J.J. Villaverde, A. De Vega, P. Ligero, C.S.R. Freire, C. Pascoal-Neto, A.J.D. Silvestre, Miscanthus x giganteus bark organosolv fractionation: fate of lipophilic components and formation of valuable phenolic by products, J. Agric. Food Chem. 58 (2010) 8279–8285.
[39] S. Minematsu, X.-Z. Wu, in: H. El-Shemy (Ed.), Vanillin and Its Detection in Air in Active Ingredients from Aromatic and Medicinal Plants, IntechOpen, 2017 Chapter 15.
[40] L.J. Jönsson, C. Martin, Pretreatment of lignocellulose: formation of inhibitory by-products and strategies for minimizing their effects, Bioresour. Technol. 199 (2016) 103–112.
[41] M. Zahedifar, Novel Ueses of Lign and Hemicellulosic Sugars from Hydolysed Lignocellulosic Materials Ph.D thesis, University of Aberdeen, UK, 1996.
[42] C. Dias, C. Silva, C. Freitas, A. Reis, T. Lopes da Silva, Effect of medium pH on *Rhodosporidium toruloides* NCYC 921 carotenoid and lipid production evaluated by flow cytometry, Appl. Biochem. Biotechnol. 179 (2016) 776–787.
[43] Q. Liu, L. Luo, L. Zheng, Lignins: biosynthesis and biological functions in plants, Int. J. Mol. Sci. 19 (2018) 335.
[44] V.S. Nogué, B.A. Black, J.S. Kruger, C.A. Singer, K.J. Ramirez, M.L. Reed, et al., Integrated diesel production from lignocellulosic sugars via oleaginous yeast, Green Chem. 20 (2018) 4349–4365.
[45] J. Yaegashi, J. Kirby, M. Ito, J. Sun, T. Dutta, M. Mirsiaghi, E. Sundstroom, A. Rodriguez, E. Baidoo, D. Tanjore, T. Pray, K. Sale, S. Singh, J. Keasling, B. Simmons, S. Singer, J. Magnuson, A. Arkin, J. Skerker, J. Gladden, *Rhodosporidium toruloides*: a new platform organism for conversion of lignocellulose to terpene biofuels and bioproducts, Biotechnol. Biofuels 10 (2017) 241.
[46] O. Ridque, Biodiesel Handling and Use Guidelines, 3rd edition, Laboratory NREL, 2006.
[47] Y. Cao, W. Liu, H. Zhang, J. Wang, M. Xian, Production of free monounsaturated fatty acids by metabolically engineered *Eschericia coli*, Biotechnol. Fuels 7 (2014) 59.