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Optimized conversion of wheat straw into single cell oils by *Yarrowia lipolytica* and *Lipomyces tetrasporus* and synthesis of advanced biofuels

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ABSTRACT

This paper deals with the optimized conversion of undetoxified wheat straw hydrolysates into microbial lipids by two oleaginous yeasts, *Yarrowia lipolytica* and *Lipomyces tetrasporus*. Wheat straw were pretreated by steam explosion at 203 °C for 300 s and hydrolysed at 20% solid-to-liquid ratio by using an enzymatic loading of 15 FPU/g substrate. The mixed wheat straw hydrolysates (WHS) contained 86 gL^{-1} glucose and 22 gL^{-1} xylose, 2.3 gL^{-1} acetic acid, 0.9 gL^{-1} furanic compounds. The fermentation process was optimized in terms of the inoculum age and density, medium composition, and bioreactor feeding strategy. In particular, the different capacity of the two yeasts to overcome the toxic effect of the biomass degradation by-products, in different inoculum ages, was deeply investigated. Two hydrolysates concentration were tested: WSH containing 86 gL^{-1} glucose and 22 gL^{-1} xylose and the diluted medium containing 40 gL^{-1} glucose and 22 gL^{-1} xylose. The results indicated that both yeasts were able to detoxify WSH and grow on undetoxified hydrolysates as effect of the intrinsic capacity to metabolize the furan derivatives. *Y. lipolytica* was able to detoxify the medium in all the investigated set-ups, while *L. tetrasporus* was able to detoxify the medium only if inoculated in the stationary phase of growth. After the process optimization in shaken flasks, the production of Single Cell Oils (SCOs) by *L. tetrasporus* was carried out in a medium-scale bioreactor of 10L obtaining lipid yield and cell content of 21% and 62%, respectively. The extracted SCOs, with high oleic and palmitic acid content, were converted into biodiesel displaying overall features in accordance with international biodiesel standards, namely ASTM and EN 14214.

1. Introduction

Due to the growing world population, the demand for global energy, food, and water is ever increasing. This has led to massive exploitation of natural resources with many environmental impacts. A paradigm shift toward more sustainable use of resources is therefore needed. In this framework, the replacement of petroleum-based compounds with renewable feedstocks is desirable [1]. The European energy directive on renewable energies, RED II, limits the use of feedstocks in competition with food chain and promotes the use of residual biomass and wastes to produce advanced biofuels [2]. Microbial oils, better known as Single Cell Oils (SCOs) are regarded as a promising raw material for the sustainable production of biofuels and chemicals due to their fatty acid composition similar to those of vegetable oils commonly used in industry [3]. Furthermore, biodiesel from microbial lipids is a particularly promising technology as it is sulfur-free, nontoxic, biodegradable [4] and its combustion emits 67% less hydrocarbon, 48% less CO_2, and 47% less particulate matter than petroleum diesel [5]. SCOs are produced by oleaginous microorganisms such as yeasts, bacteria, fungi, and microalgae. The yeasts are among the most promising microorganisms for the production of oils since, depending on the feedstocks, they can accumulate lipids up to more than 70% of their dry cell weight, have high growth rates, are easily cultivable, and use less land and water than vegetable oils [6]. Furthermore, oleaginous yeasts can grow on several carbon sources, such as simple sugars (e.g. glucose and fructose), lactose, sucrose [7], whey [8], volatile fatty acids [9], and industrial waste [10].

Due to the high production cost of SCOs, about 4.24 $/kg [11], only SCO with high polyunsaturated fatty acids were produced so far at the industrial scale, while the industrial production for biofuels or green chemistry applications is far behind [12].

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Nomenclature

WSH	Wheat Straw Hydrolysate
WSH80	High sugars medium
WSH40	Low sugars medium
SP	Stationary Phase
EP	Exponential Phase
SCOs	Single Cell Oils
DCW	Dry Cell Weight
FAs	Fatty Acids
PUFAs	Polyunsaturated Fatty Acids
FAMEs	Fatty Acids Methyl Esters
SYN	Synthetic Medium
5-HMF	5-Hydroxymethylfurfural
HCV	High Calorific Value
LCV	Low Caloric Value
CN	Cetane Number
IV	Iodine Value
CFPP	Cold-Filter Plugging Point

The carbon source is an important cost item in determining the overall lipids production cost. In this sense, it is expected that the utilization of renewable low-cost feedstocks, along with an increased bioconversion performance, boosts the economic value of the SCO production process [13]. Several types of waste and low-cost carbon sources, including olive mill wastewater, by-products of the dairy industry, and food processing, have been used as feedstock to produce microbial lipids [12]. Lignocellulosic biomasses hold many advantages since they are abundant and many technologies are available to convert them into sugars. Due to their recalcitrant nature, lignocellulosic biomasses require pretreatments to favour the breakdown of the lignin structural matrix and favour the subsequent enzymatic hydrolysis of polysaccharides to fermentable sugars. Biomass pretreatment followed by enzymatic hydrolysis leave an unconverted lignin residue, hydrolytic lignin, that can be valorised for the production of several green chemicals including carbon fibres, aromatic monomers, phenolic resins and polyols [14]. One of the main problems related to the use of lignocellulosic sugars for microbial conversion is the concentration of toxic compounds generated during the pre-treatment process, mostly 5-hydroxymethylfurfural (5-HMF), furfural, and acetic acid and lignin derived compounds. These compounds could inhibit yeast growth and affect lipid synthesis, therefore, purification processes are required [15]. Previous investigations demonstrated that in many microorganisms the growth phase of inoculum could importantly affect the yeast tolerance [16,17]. This was never investigated so far for oleaginous yeasts.

The present paper was aimed at the conversion of undetoxified wheat straw hydrolysate into SCOs by two oleaginous yeasts, *Lipomyces tetrasporus* and *Yarrowia lipolytica*. *Y. lipolytica* was chosen as control strain as it is characterized by flexible metabolism, high-stress tolerance, versatility of growth on different carbon sources, and its GRAS status (generally recognized as safe) [18]. As regards *L. tetrasporus*, it has been less investigated than other yeasts despite its interesting potential for lipids accumulation [16]. The fermentation process was optimized in terms of the inoculum age, medium composition and bioreactor feeding strategy. After the process optimization in shaken flasks, the production of SCOs by *L. tetrasporus* was carried out in a medium-scale bioreactor and the extracted SCOs were used to produce biodiesel.

2. Materials and methods

2.1. Yeast strains and pre-inoculum preparation

Lipomyces tetrasporus Li-0407 (DSM 70314, DSMZ, Germany) and *Yarrowia lipolytica* ATCC 46483 (DSM 8218, DSMZ, Germany) were purchased from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures. The yeasts were preserved on YPD agar slant (Sigma-Aldrich) at 4 °C and sub-cultured every week. The pre-inoculum was prepared by inoculating a loopful of each strain into 100 mL YPD Broth in 500 mL-Erlenmeyer flasks, and incubated at 27 °C under agitation (200 rpm). For the evaluations of influence of the metabolic phase of inoculum, each yeast was harvested, on the basis of previous test (data not shown), in exponential phase (48 h for *Y. lipolytica* and 70 h for *L. tetrasporus*) and at the beginning of the stationary phase (96 h for *Y. lipolytica* and 120 h for *L. tetrasporus*). After incubation a precise volume of cell suspension, evaluated by spectrophotometric quantification at 600 nm, was transferred to achieve the desired initial cells concentration of $3.6 * 10^6$ and $6.2 * 10^6$ CFU/mL, corresponding to OD 5 and OD 10. The growth media were sterilized (121 °C, 15 min) before inoculation.

2.2. Growth in synthetic media

In the first step, *Lipomyces tetrasporus* and *Yarrowia lipolytica* were grown on 2 synthetic media (SYN 40 and SYN 80), with same chemical composition, with exception of sugar concentration (low and high-sugar medium, respectively). The SYN media was prepared with a carbohydrate composition similar to the WSH but without the inhibiting compounds. The culture media composition (SYN 80) was the following: glucose 86.4 gL^{-1}, xylose 22.1. gL^{-1}, galactose 0.9 gL^{-1}, arabinose 1.7 gL^{-1}, yeast extract 4.75 gL^{-1}, $MgSO_4 \bullet 7H_2O$ 1.5 gL^{-1}, $MnSO_4 \bullet H_2O$ 0.004 gL^{-1}, $CuSO_4 \bullet 5H_2O$ 0.001 gL^{-1}, KH_2PO_4 2 gL^{-1}, Na_2HPO_4 0.3 gL^{-1}, and $ZnSO_4$ 0.04 gL^{-1} (Sigma Aldrich, Italy). SYN 40 showed a glucose concentration halved compared to SYN 80, equal to 40.2 gL^{-1}. For all tests, the initial C/N ratio was kept at 85 mol/mol, pH was set at 5.5 and was not adjusted through the fermentation. Temperature and stirring were set at 27 °C and 200 rpm, respectively. All the experiments were conducted in triplicate.

2.3. Characterization and pretreatment of wheat straw

The wheat straw was collected from agricultural sites in Southern Italy. The residues were divided into stocks of around 10 kg and stored indoors. Prior to the pretreatment, the biomass had a dry matter content of 91.9 ± 0.2%. After grinding at 50 mesh, air-dried wheat straw was analysed for extractives, carbohydrate, lignin, and ash content by the methods developed by Sluiter et al., [19]. The raw material contained (%): 38.4 ± 3.2 glucan, 16.7 ± 1.1 xylan, 3.1 ± 0.2 arabinan, 1.2 ± 0.1 galactan, 20.6 ± 1.1 acid-insoluble lignin, 6.2 ± 0.1 ash, 4.3 ± 0.3 extractives. To clarify the components of the hydrolysate was used HPIC (DIONEX ICS2500, Germany) system equipped with a Nucleogel Ion 300 OA operating at 60 °C with 10 mN H_2SO_4 solution as mobile-phase (0.4 mL min^{-1}). The detector was a Shodex RI101 refractive index. Each analysis was carried out in duplicate.

The biomass degradation by-products were quantified using the HP1100 system (Agilent, USA), equipped with a RP18 5 μm LiChroCart 250 × 4mm (Agilent, USA) column operating at 50 °C with Milli-Q-water/acetonitrile as mobile-phase (1 mL min^{-1}) and a diode array detector. Quantification was carried out at two different wavelengths, 210 and 280 nm.

Steam explosion has been recognized as one of the most effective and versatile pretreatments for the fractionation of biomass into fermentable sugars and residual lignin, also through the use of acid or base catalysts. The process conditions for the pretreatment of wheat were based on previous papers with similar set-up [20]. In particular, before the pretreatment, biomass was crumbled to particles size in the range 1.7–5.6 mm and was soaked in a dilute H_2SO_4 solution (0.05 M) for 10 min in 10% s/L (w/v) suspension. The resulting acid load on raw material was measured following titration of the impregnation liquid and was found to be 1.4% (w/w). The pretreatment was carried out at 203 °C for 5 min

similarly to process parameters optimized by other authors during the acid catalysed steam explosion of wheat straw in order to obtain a high hydrolysability of the cellulose [21,22]. The pretreated product was filtered in order to separate the solids from the soluble fraction, containing mainly hemicellulose. The composition of the pretreated product, consisting of a solid fraction rich in cellulose and a liquid fraction containing soluble hemicellulose, was quantified according to Sluiter et al., [19]. Due to the steam condensation, biomass after the pretreatment had high moisture content. It was then squeezed to separate the solids and after that remixed up to achieve the target solids concentration of 20% for the enzymatic hydrolysis.

2.4. Enzymatic hydrolysis of the pretreated wheat straw

The enzymatic hydrolysis of the pretreated wheat straw was carried out in a 10 L stirred bioreactor (Braun Biotech International, Germany) equipped with a helical impeller. Cellic™ CTec2, kindly provided by Novozymes (Denmark) was added in a dosage of 15 Filter Paper Unit (FPU) per gram of insoluble glucan. The filter paper activity of the enzymatic blend was 190 FPU/g, determined according to the Ghose method [23]. The biomass slurry was hydrolysed at pH 4.8, 50 °C, 180 rpm for 90 h. The temperature was then raised at 100 °C for few minutes to denature the enzymatic proteins. A cellulose conversion of around 90% was obtained. Hydrolysate was then added with a solution of nutrients and sterilized at 121 °C for 20 minutes. The sterilized solution was inoculated with yeast for the fermentative processes.

2.5. Up-scaling of bioconversion process in 10 L bioreactor

The production of SCOs was scaled-up by using a 10 L stirred tank bioreactor; this step was performed by inoculating *L. tetrasporus* strain and by using growth conditions optimized in the first phases. The pH was set at 5.5 and was then automatically adjusted through the addition of NaOH 4N and H_2SO_4 4M. The dissolved oxygen concentration was maintained at least above 40% through the stirring speed and air flow. The process was carried out in the fed-batch mode. The initial working volume was 6500 mL, subsequently increased to 7500 mL after the addition of two concentrated feeds of 500 mL each. To avoid medium dilution during the fed-batch operations, hydrolysate was concentrated in a laboratory vacuum system at 7 kPa, 180 rpm, and 60 °C for 5 h, reducing a volume of 13.5 L added with microelements to a final volume of 1000 mL.

2.6. Lipids extraction and Fatty acids methyl esters (FAMEs) determination

Lipid extraction from yeast biomass recovered at the end of bioconversion processes was performed using the protocol previously described [16].

According to the method of [24], the microbial lipids were transmethylated. The methyl esters profiles were determined by GC analyses carried out on Agilent GC7890A gas chromatograph, equipped with OMEGAWAX 250 (Agilent) capillary column (30 m × 0.32 mm x 0.50 μm) and a flame ionization detector. The oven temperature was programmed at 80 °C for 11 min, from 80 °C to 180 °C at a rate of 20 °C/min and held at 180 °C for 22 min. Helium was the carrier gas (1 mL min^{-1}). Split ratio was 1:19 (v/v). Identification of methyl esters was performed comparing the peaks retention times to FAMEs standards mix, C8– C24 (CRM 18918 Sigma-Aldrich).

Glyceryl triundecanoate was used as internal standard for evaluating the extraction yields of the lipids while methyl heptadecanoate was used as internal standard for the quantification of FAME by chromatographic analysis.

2.7. Biodiesel synthesis and characterization

The lipid obtained above was transformed into biodiesel according to the classical transesterification method [25]. In brief, 50 g of the oven dried (60 °C overnight) microbial oil were mixed with 10 g NaOH-methanol solution (MeO^-/MeOH 0.05:1) and placed in a magnetic agitator at 300 rpm at 60 °C for 60 min. The resulting organic phase was separated by centrifugation at 9000*g* for 10 min and subsequently washed for three times with hot water, after that the mixture was maintained in an oven at 50 °C overnight. The obtained organic phase was used as microbial biodiesel. The FAMEs profile was analysed according paragraph 2.6.2. Density (ρ) was measured with a density meter Anton-Paar (model DMA48). Kinematic viscosity (ν) of biodiesel was determined using the manual calibrated glass capillary viscometer Cannon Fensche S490 size 75, at 40.00 °C (UNI-EN-ISO 3104–2000).

The water content was determined by a Karl–Fischer automatic Titrator Mettler DL18 (EN ISO 12937–2000). High calorific value (HCV) was analysed using a calorimeter bomb (IKAC5000).

Low calorific value (LCV) was calculated from HCV using the Dulong equation according to Erdoğan et al [26]:

$$LCV = HCV\text{-}\ (22.5 * H\ \% / 100)$$

where H% is the relative percentage hydrogen in the biodiesel determined by elementary analysis (VARIO MACRO CUBE). Iodine Value (IV), Cetane Number (CN) and Cold Filter Plugging Point (CFPP) were calculated using semi-empirical formulas as reported in Viola et al. [27].

2.8. Process yields calculation

The different process yields were calculated by the following equations:

1) DCW yield: gram of dry cell weight per gram of sugar consumed * 100;
2) Lipid yield: gram of lipids produced per gram of sugar consumed * 100;
3) Productivity: gram liter of biomass produced per time of process;
4) Lipid content: gram of lipids produced per gram of dry cell weight * 100;
5) Mannitol yield: gram of mannitol produced per gram of sugar consumed * 100;

2.9. Statistical analysis

Data of yeast performance in SYN and WSH media under different conditions were analysed by one- or two-way analysis of variance (ANOVA). Levene's test ($p < 0.05$) was used to verify the variance homogeneity. Tukey's test was used to compare the mean values between different conditions. Data obtained by analysis of main fatty acid composition of lipid extracted by the two strains were submitted to classical clustering analysis, by using Ward's algorithm and Euclidean similarity index. The software used for the statistical analyses was PAST ver. 3.26 [28].

3. Results and discussion

3.1. Yeast performance in synthetic media under different carbon concentrations, inoculum levels and metabolic phase of growth

Previous studies, reporting the use of *Yarrowia lipolytica* and *Lipomyces tetrasporus* in the conversion of several carbon sources for the production of SCOs [29,30], showed that the process yields are affected by different factors, such as the yeast strain, type and concentration of the carbon sources, and process set-ups. In a recent publication regarding the conversion of cardoon hydrolysates by *L. tetrasporus* and

C. curvatus, we found that the growth phase of the inoculum affected the strain ability to metabolize some degradation products by converting them into non-toxic molecules [16]. Obviously different hydrolysates could have different inhibition thresholds due to different specific compositions thus requiring a fine-tuning of the fermentation set-ups. Although cardoon and wheat straw had a similar composition of sugars and representative inhibitory compounds there may be some inherent differences. More in general, given the complexity of the lignocellulosic matrix, additional degradation products that were not identified are likely present in the hydrolysate. Different lignin-derived products were found by Vergara et al. [31], in wheat straw acid hydrolysates compared to cardoon. Even if enzymatic hydrolysis used in this work is a milder process than acid hydrolysis, the presence of different lignin fragments cannot be excluded that could exert a different overall inhibitory effect. In the present paper, *Y. lipolytica* and *L. tetrasporus* were first tested by using synthetic media with sugar compositions similar to wheat straw hydrolysate. This is important to explore the kinetics of glucose and xylose uptake and explore the conversion yield toward the biomass, lipids, and polyols production.

The trials were carried out in high and low-sugar media (SYN 80 and SYN 40, respectively), by using two metabolic phases of the inoculum, Stationary Phase (SP) and Exponential Phase (EP), and two inoculum sizes (corresponding to OD 5 and OD 10).

In order to study the significant differences between two metabolic phases of the inoculum, SP and EP, and two sugar concentrations (SYN 80 and SYN 40, respectively), the process yields were statically analysed by ANOVA. The analysis, reported in Table 1, showed that there were no statistically significant differences for the *Y. lipolytica* strain with inoculum density OD 10. Only for the biomass yield value (Y DCW) in SP inoculum phase, a difference ($p < 0.05$) between SYN 80 and SYN 40 was appreciable. On the contrary, for the OD 5 inoculum density, in the EP inoculum phase, a significant difference was shown between SYN 80 and SYN 40 both for the value Y DCW and Y lipids. *Y. lipolytica* produced a biomass yield in the range 47–63% higher than *L. tetrasporus*, which ranged between 24 and 38% (Table 1).

The highest biomass production of 63% was obtained with *Y. lipolytica* inoculated in EP (Fig. 1 a, orange bar) SYN 40 and OD5. Under the same conditions, *Y. lipolytica* produced the best lipid yield, corresponding to 13.5% lipids (Fig. 1 b, orange bar). Similar yields were obtained in the same inoculum phase, EP, and medium concentration, SYN 40, at higher inoculum size, OD 10 (Fig. 1 b, orange bar) corresponding to a lipids cell content of 26% (Fig. 1 c, orange bar). The SP inoculum showed even higher biomass yields (DCW) compared to EP but only in media with the highest sugar concentrations, namely SYN 80 (Fig. 1 a, blue bar).

Process yields observed for the *Y. lipolytica* strain were comparable to the results of Enshaeieh et al. [32], relevant to the conversion of synthetic media containing 95 gL^{-1} glucose. Moderate amounts of mannitol in the range 1.3–5.8 gL^{-1} were also detected. Some authors described the production of citric acid by *Y. lipolytica* strain DSM 8218 [33], erythritol, mannitol, and arabitol using different *Y. lipolytica* strains [34], isocitrate, using the strain VKM Y-2373 [35], alpha-ketoglutarioc acid using the strain A8 [36], and acetic acid using 6 different *Y. lipolytica* strains [37]. Although the production of mannitol was already observed in several *Y. lipolytica* strains, no specific data were reported so far for the strain DSM 8218 grown on sugars and the results presented in this paper enlarge the knowledge base of this microorganism. No statistical correlations with the process conditions emerged, therefore further analyses are necessary to assess the correlation between the formation of these metabolites and specific set-ups.

In all conditions, *L. tetrasporus* produced from 1.5 to 2-fold more lipids than *Y. lipolytica,* range 13.5–23.2 and 8.3–13.5%, respectively (Table 1).

By considering OD10 as inoculum level, both the DCW and lipid yield were significantly influenced by the sugar concentration ($p < 0.05$), (Table 1). As showed in Fig. 1a and 1b, green bar, the highest DCW yield (38.8%) and lipid yield (23%) were obtained with SP cells in SYN 40 and the highest inoculum level (OD 10), whereas the highest lipid content (64%) was obtained in SYN 40 inoculated with EP cells, OD 10 (Fig. 1 c, yellow bar). In addition to the production of lipids, the production of small quantities of two secondary products was observed, one of which was identified as mannitol. This is in agreement with the analysis of secondary metabolites by *L. tetrasporus* recently published Caporusso et al., [16]. Further analyses are necessary to assess the correlation between the formation of these metabolites and specific process conditions.

L. tetrasporus in some conditions showed productivity up to 40% higher than *Y. lipolytica* (Table 1) and both yeasts showed higher productivity in low-sugar medium. In SYN 80, both yeasts were unable to completely consume glucose and at the end of the process more than 50% of xylose was still available in the medium (only for SP cell of *Y. lipolytica* about 83%). Conversely, in SYN 40 only for *L. tetrasporus* less than 50% xylose was present in the medium at the end of the process (data not shown). Furthermore, galactose and arabinose were available in the hydrolysate in lower concentration with respect to glucose and xylose, below 2 gL^{-1}. Arabinose was not converted, galactose and glucose were consumed simultaneously and the maximum contribution to the lipids production from galactose was negligible, less than 5%.

Table 1
Performance of Yarrowia lipolytica and Lipomyces tetrasporus on synthetic media SYN 80 and SYN 40, inoculated in Stationary Phase (SP) and Exponential Phase (EP), at two inoculum levels (OD 10 and 5). For each parameter and each strain, data with different superscript letters mean significant differences ($p \leq 0.05$) among samples with the same inoculum size (OD 10 and OD 5 separately).

	Yarrowia lipolytica ATCC 46483							
	OD10				OD5			
	SP-SYN 80	SP-SYN 40	EP-SYN 80	EP-SYN 40	SP-SYN 80	SP- SYN 40	EP-SYN 80	EP-SYN 40
Y DCW %	60 ± 2^{a}	49 ± 3^{b}	55 ± 3^{ab}	52 ± 2^{ab}	54 ± 3^{a}	47 ± 1^{a}	51 ± 2^{a}	63 ± 3^{b}
Productivity	0.05 ± 0.01	0.05 ± 0.01	0.06 ± 0.01	0.07 ± 0.01	0.05 ± 0.01^{a}	0.05 ± 0.01^{ab}	0.06 ± 0.01^{a}	0.08 ± 0.00^{ac}
Y lipids %	11 ± 2	10.2 ± 0.1	12 ± 5	13.5 ± 0.8	9.9 ± 0.5^{a}	8.3 ± 0.2^{a}	9 ± 1^{a}	13.5 ± 0.3^{b}
Lip/DCW %	18.7 ± 0.5	22 ± 5	21 ± 7	26 ± 3	19 ± 2	18 ± 1	19 ± 1	21 ± 1
Lipomyces tetrasporus Li-0407								
	SP-SYN 80	**SP-SYN 40**	**EP-SYN 80**	**EP-SYN 40**	**SP-SYN 80**	**SP- SYN 40**	**EP-SYN 80**	**EP-SYN 40**
Y DCW %	27.6 ± 0.3^{a}	38.8 ± 0.3^{b}	25.9 ± 0.3^{ac}	30 ± 2^{ad}	24 ± 2^{a}	28.8 ± 0.8^{ab}	25 ± 2^{a}	$35^{b} \pm 3^{b}$
Productivity	0.08 ± 0.01^{a}	0.09 ± 0.01^{b}	0.07 ± 0.01^{a}	0.07 ± 0.01^{a}	0.07 ± 0.01	0.07 ± 0.01	0.07 ± 0.01	0.08 ± 0.01
Y lipids %	15.9 ± 0.7^{a}	23 ± 2^{b}	14 ± 1^{a}	19.3 ± 0.4^{b}	14.1 ± 0.2^{a}	17.4 ± 0.7^{ac}	13.5 ± 0.1^{ad}	21 ± 2^{b}
Lip/DCW %	58 ± 2	60 ± 7	54 ± 4	64 ± 5	60 ± 4	60.4 ± 0.9	54 ± 4	61.3 ± 0.4

Dry Cell Weight yield (Y DCW) was expressed as a gram of DCW per gram of sugar consumed * 100 Productivity was expressed as a gram of DCW per process time Lipid yield (Y lipids) was expressed as gram of Lipids per gram of sugar consumed * 100 Intracellular lipids (Lip/DCW) was expressed as gram of Lipids in function of DCW gram *100.

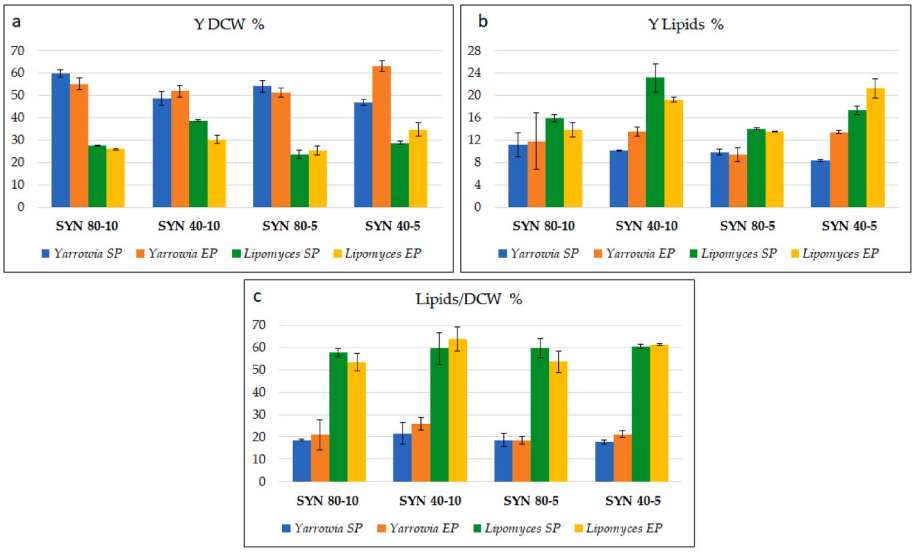

Fig. 1. *Production of biomass (Y DCW) and lipids (Y Lipids) as a function of the sugars consumed. Intracellular lipids accumulation (Lipids/DCW) calculated as lipids produced as a function of cell biomass* Yarrowia lipolytica inoculated in Stationary Phase, SP (blue bar) and Exponential Phase, EP (orange bar), Lipomyces tetrasporus inoculated in Stationary Phase SP, (green bar) and Exponential Phase, EP (yellow bar). The concentration of the medium (SYN80 or SYN40) and the quantity of inoculum (OD 10 or 5) are reported on the abscissas.

Many oleaginous microorganisms are able to grow on different carbon sources, including several C5 sugars (xylose, arabinose, etc …) [38,39]. The study of yeast metabolism on C5 sugars is important for the complete valorisation of all lignocellulosic biomass fractions. Furthermore, it has also been found that using xylose as a carbon source for lipid production may even be more performance than glucose in some cases. The theoretical maximum lipid yield from glucose is 0.32 g/g, while the theoretical maximum yield from xylose is about 0.34 g/g in the case of the phosphoketolase pathway and 0.30 g/g for the pentose phosphate pathway [12]. Xylose consumption typically starts after glucose depletion or in correspondence of specific internal sugars concentration ratio.

The higher affinity for glucose with respect to other carbon sources available in the medium is a well-known process, already documented for many oleaginous species due to the carbon catabolite repression [40]. This mechanism leads, in some cases, to diauxic growth phenomena that affect the metabolism of the secondary sugars and further limits the conversion efficiency during the fermentative process [41]. Some authors also found that the slow transport rate of xylose limits the downstream pathway; consequently, the enhancement of the transport rate is necessary for improving the cell growth rate and the xylose conversion efficiency [42,43].

Table 2 includes the detailed FAs composition of the triglycerides achieved through the investigated set-ups. The most common FAs produced by oleaginous yeasts are oleic acid (C18:1), palmitic acid (C16:0), linoleic acid (C18:2), stearic acid (C18:0) and low quantity of lauric acid (C12:0), myristic acid (C14:0), palmitoleic acid (C16:1) and linolenic acid (C18:3) [4]. The two yeasts had a different lipid profile. *Y. lipolytica* produced about 40% oleic acid, followed by about 15% linoleic and palmitic acids, about 10% stearic and palmitoleic acids. An interesting result is the production of small quantities of short-chain FAs, namely lauric and myristic acids, and PUFAs, in particular linolenic acid. The FAs profile in *Y. lipolytica* was affected by process parameters, such as

Table 2
Main fatty acids produced by *Yarrowia lipolytica* and *Lipomyces tetrasporus* grown in synthetic media SYN 80 and SYN 40, inoculated in Stationary Phase (SP) and Exponential Phase (EP), at two inoculum levels (OD 10 and 5). The values have been normalized and correspond to % of the total fatty acids produced.

	SP OD 10		EP OD 10		SP OD 5		EP OD 5	
Y. lipolytica	SYN 80	SYN 40	SYN 80	SYN 40	SYN 80	SYN 40	SYN 80	SYN 40
C12:0	4.5 ± 0.3	2.1 ± 0.2	1.5 ± 0.1	0.0 ± 0.0	3.4 ± 0.2	2.7 ± 0.2	2.1 ± 0.2	1.7 ± 0.1
C14:0	4.4 ± 0.3	1.5 ± 0.1	3.2 ± 0.2	2.0 ± 0.1	2.2 ± 0.1	2.1 ± 0.1	0.0 ± 0.0	3.2 ± 0.2
C16:0	15 ± 1	21 ± 1	20 ± 1	16 ± 1	17 ± 1	18 ± 1	15 ± 1	20 ± 2
C16:1	10.1 ± 0.8	11 ± 1	11.4 ± 0.9	7.4 ± 0.6	8.7 ± 0.5	16 ± 1	12.5 ± 0.9	9.3 ± 0.8
C18:0	10.5 ± 0.9	10.9 ± 0.9	10.5 ± 0.8	13 ± 1	10.±0.7	7.9 ± 0.5	14 ± 1	10.4 ± 0.8
C18:1	38 ± 2	43 ± 2	39 ± 2	45 ± 2	45 ± 3	40 ± 3	43 ± 3	46 ± 3
C18:2	15 ± 1	10 ± 1	12.7 ± 0.8	15.1 ± 0.2	11.3 ± 0.9	9.4 ± 0.8	13.4 ± 0.3	9.3 ± 0.78
C18:3	1.20 ± 0.09	0.80 ± 0.05	1.10 ± 0.06	0.40 ± 0.01	0.90 ± 0.05	0.50 ± 0.03	0.00 ± 0.00	0.00 ± 0.00
C20:0	0.00 ± 0.00	0.40 ± 0.02	0.70 ± 0.03	0.00 ± 0.00	1.00 ± 0.06	1.30 ± 0.05	0.00 ± 0.00	0.00 ± 0.00
C22:0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
C24:0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
L. tetrasporus	**SYN 80**	**SYN 40**	**SYN 80**	**SYN 40**	**SYN 80**	**SYN 40**	**SYN 80**	**SYN 40**
C12:0	0.39 ± 0.02	0.39 ± 0.01	0.35 ± 0.01	0.30 ± 0.01	0.24 ± 0.01	0.33 ± 0.01	0.33 ± 0.02	0.29 ± 0.01
C14:0	0.54 ± 0.04	0.53 ± 0.03	0.50 ± 0.04	0.53 ± 0.03	0.47 ± 0.03	0.48 ± 0.03	0.60 ± 0.04	0.55 ± 0.04
C16:0	28 ± 2	28 ± 2	26 ± 2	27 ± 2	27 ± 2	28 ± 2	26 ± 2	27 ± 2
C16:1	1.5 ± 0.1	1.4 ± 0.1	1.5 ± 0.1	1.5 ± 0.1	1.5 ± 0.1	1.5 ± 0.1	1.4 ± 0.1	1.8 ± 0.1
C18:0	24 ± 2	24 ± 2	24 ± 2	24 ± 2	23 ± 2	21 ± 2	24 ± 2	21 ± 2
C18:1	43 ± 2	42 ± 2	44 ± 3	42 ± 3	44 ± 3	44 ± 2	43 ± 2	46 ± 3
C18:2	0.63 ± 0.04	0.67 ± 0.03	0.69 ± 0.02	0.50 ± 0.03	0.68 ± 0.05	0.64 ± 0.04	0.59 ± 0.04	0.64 ± 0.03
C18:3	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
C20:0	0.95 ± 0.05	0.90 ± 0.04	0.93 ± 0.06	0.87 ± 0.06	0.99 ± 0.06	0.91 ± 0.05	0.96 ± 0.06	0.89 ± 0.05
C22:0	0.00 ± 0.00	0.79 ± 0.05	0.44 ± 0.04	0.53 ± 0.03	0.81 ± 0.07	0.65 ± 0.05	0.88 ± 0.07	0.47 ± 0.03
C24:0	1.6 ± 0.1	1.7 ± 0.1	1.6 ± 0.1	1.6 ± 0.1	1.4 ± 0.1	1.5 ± 0.1	1.5 ± 0.1	1.6 ± 0.1

sugar content of media, growth and inoculum level.

As regards *L. tetrasporus*, a low influence of the process parameters on the lipid profile was observed. Differently from *Y. lipolytica*, *L. tetrasporus* produced about 40% oleic acid, followed by about 25% stearic and palmitic acids, and a low quantity of other fatty acids. Interestingly, *L. tetrasporus* did not produce PUFAs but produced traces of long-chain FAs, namely arachidic acid (C 20:0), behenic acid (C 22:0), and lignoceric (C 24:0) acid. Similar lipid profiles were also found by Sitepu et al., [44], whit the strain *L. lipofer* UCDFST 78-19T under similar conditions or by Dien et al. [45] with *L. tetrasporus* Y11562 grown on undetoxified hydrolysate of Douglas fir forest residues.

On the whole, the lipids profile demonstrated that both yeasts could be used for the production of microbial oils suitable for different target applications and that the distinctive metabolic pathways could be overexpressed to foster for instance a higher production of the minor components with high added value.

3.2. Wheat straw hydrolysates

Biomass pretreatment is necessary to increase the polysaccharides' accessibility in the enzymatic hydrolysis. The combined effect of the saturated water vapor and the acid catalyst promoted the autohydrolysis of the acetyl groups from carbohydrates and increased the biomass hydrolysability [46,47]. Enzymatic hydrolysis of wheat straw was already investigated by several authors [48–50]. The glucose yields typically range from 80% to 98%, depending on the technology and pretreatment conditions, the type of enzymes and their dosage, and the load of solids. In particular, Horn et al. [49], obtained a hydrolysis yield of 88% with low solids load (5%) by using an enzymatic blend no longer in use, such as Celluclast/Novozym. Kontogianni et al. [48], tested the enzymatic hydrolysability of exploded wheat straw with a higher solids load (around 12%) by using the more recent enzymatic blend CTec2, obtaining a quantitative hydrolysis yield (98%). Furthermore, Alvira et al. [50], tested a solid load of 15% with a hydrolysis yield of around 80%. In the present investigation acid-catalysed steam exploded wheat straw was treated with CTec2 at 20% biomass loads. After the steam explosion process, the pretreated product consisted of a cellulose-rich solid fraction and a liquid fraction containing hemicellulose (Table 3). The hemicellulose-rich fraction contained several degradation products, including phenol-like molecules from the lignin depolymerization, that can reduce the hydrolysis yield as effect of enzymatic inhibition or adsorption on cellulose. The steam pretreated pulp had an overall dry matter of 12.5 ± 0.8% with the following composition (%): 35.0 ± 1.4 glucan, 18.2 ± 0.9 xylan, 1.4 ± 0.2 arabinan, 0.9 ± 0.1 galactan, 21.2 ± 1.5 insoluble acid lignin, 1.95 ± 0.24 acetyl groups, 0.15 ± 0.02 5-HMF, 0.74 ± 0.04 furfural, calculated as % of the pretreated biomass dry matter. The steam-pretreated product was hydrolysed at high DM content to achieve mixed hydrolysates. The final glucose and xylose yields were 89% and 71% respectively. The detailed composition of the hydrolysate (WSH 80) is summarized in Table 3. The diluted hydrolysate was achieved from the WSH 80 through the addition of the hemicellulose in the ratio 1:1 (WSH 40).

3.3. Yeast performance in hydrolysate under different concentrations, inoculum levels and metabolic phase of growth

The main problem hindering the efficient conversion of the lignocellulosic sugars as carbon source for microbial processes is the presence of toxic molecules derived from the lignocellulose degradation. Biomass degradation by-products often raise the need of introducing detoxification steps by chemical-physical methods before microbial conversion [51]. Despite the benefits over increased sugars fermentability, the detoxification methods are often costly. Furthermore, there is no specific detoxification method for all situations and each method has a specific set of advantages and disadvantages. In situ biological detoxification is based on the capability of some microorganisms to develop an adaptive response when subjected to external stresses thus resulting more tolerant than others. An investigation from Sitepu et al. [5] reported a wide assessment of the inhibitor tolerance of 45 oleaginous yeasts species. Differently, Kim et al. [52] improved the furfural tolerance and the biological detoxification of *Y. lipolytica* by overexpressing the aldehyde dehydrogenase family proteins. The possibility of regulating the yeast response through the inoculum age, as proposed in the present work, offers a versatile tool to manage the hydrolysates toxicity. Several authors studied the toxicity of different molecules toward oleaginous yeasts [5,53–55]. The inhibition threshold is the result of many concurring factors, including the microbial strain and the synergistic effect of different microbial inhibitors. The detailed characterization of the biomass hydrolysates is complex because it could contain many oligomeric products that are often not detected by HPLC analysis and that can contribute to lower the toxicity thresholds associated with specific molecules, such as furan derivatives or acids. For this reason, the toxicity threshold of biomass hydrolysates needs to be assessed on the whole since the uptake and metabolism of degradation by-products could significantly vary from one hydrolysate to another [53,56,57].

Considering the complexity of the lignocellulosic hydrolysates, 5-HMF and furfural were investigated as representative inhibitors since they are likely the most abundant and also the metabolic products are known and can be monitored during the process. The main scope of the present experimental design for the process optimization was to maximize the lipid production by *L. tetrasporus* and *Y. lipolytica* in undetoxified hydrolysates. Furan compounds were used as markers of the biodetoxification of the yeast cells in the tested conditions.

The ANOVA analysis on wheat straw hydrolysate (Table 4) showed that the *Y. lipolytica* metabolism in terms of Y DCW and productivity, was significantly influenced for both OD 10 and OD 5 by the inoculation phase, EP and SP, but not by sugars concentration. On the other hand, the production of lipids did not show statistically significant differences ($p < 0.05$) for the inoculation phase and sugar concentration. *Y. lipolytica* confirmed its higher biomass yield 30–61% compared to *L. tetrasporus* 0–30% (Table 4).

The productivity of *Y. lipolytica* remained unchanged compared to SYN media only for SP inoculation, whereas the inoculum of EP cells yielded lower productivity in comparison to SYN medium. This emphasizes that cells in the EP inoculum had a greater sensitivity to the toxicity of the hydrolysate. Furfural usually increases the lag phase in a linear matter, while 5-HMF usually reduces the growth rate [53]. The 51-30 *Yarrowia* strain resulted sensitive to furfural as it was inhibited when grown on a medium containing 0.5 gL^{-1} of furfural [5], whereas other authors [53] showed the ability of *Yarrowia* W29 strain to grow in presence of furfural concentrations up to 2.9 gL^{-1}, although after a prolonged lag phase. In the present work, the analysis of the furfural and 5-HMF revealed that the microorganism consumed both the compounds

Table 3
Composition of hydrolyzate obtained from steam explosion and enzymatic hydrolysis of wheat straw (WSH 80). WSH 40 was obtained from WSH 80 by *dilution with hemicellulose in ratio 2:1. Hemicellulose was the liquid fraction obtained from steam pretreated wheat straw.*

	Glucose gL^{-1}	Xylose gL^{-1}	Galactose gL^{-1}	Arabinose gL^{-1}	Acetic acid gL^{-1}	5-HMF $mgKg^{-1}$	Furfural $mgKg^{-1}$
WSH 80	86 ± 7	22 ± 2	0.9 ± 0.1	1.7 ± 0.1	2.3 ± 0.2	145 ± 8	705 ± 10
WSH 40	40 ± 2	22 ± 1	0.9 ± 0.2	1.8 ± 0.1	2.3 ± 0.3	152 ± 7	700 ± 13
Hemicellulose	1.0 ± 0.1	16 ± 1	0.7 ± 0.1	1.4 ± 0.1	1.9 ± 0.3	150 ± 5	723 ± 11

Table 4
Growth performance of Yarrowia lipolytica and Lipomyces tetrasporus on wheat straw hydrolysate, WSH 80 and WSH 40, inoculated in Stationary Phase (SP) and Exponential Phase (EP), by using high and low inoculum levels (OD 10 and OD 5, respectively). For each parameter and each strain, data with different superscript letters mean significant differences ($p \leq 0.05$) among samples with the same inoculum size (OD 10 and OD 5, separately).

	Yarrowia lipolytica ATCC 46483							
	OD10				OD5			
	SP-WSH 80	SP-WSH 40	EP-WSH 80	EP-WSH 40	SP-WSH 80	SP-WSH 40	EP-WSH 80	EP-WSH 40
Y DCW%	61 ± 2^{a}	58 ± 4^{a}	38 ± 3^{b}	39 ± 2^{b}	56 ± 4^{a}	47 ± 2^{ab}	42 ± 4^{bc}	30 ± 2^{c}
Prod	0.05 ± 0.01^{a}	0.05 ± 0.01^{a}	0.04 ± 0.01^{b}	0.04 ± 0.01^{b}	0.05 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.01
Y lip%	4.7 ± 0.8	5.3 ± 0.5	3.8 ± 0.01	5.3 ± 0.5	5.2 ± 0.5^{a}	4.0 ± 0.2^{ab}	4.0 ± 0.3^{ab}	3.8 ± 0.3^{b}
Lip/DCW %	7.8 ± 0.1^{a}	9.1 ± 0.3^{b}	9.9 ± 0.3^{b}	13.4 ± 0.5^{c}	9.4 ± 0.5^{a}	8.5 ± 0.2^{a}	9.9 ± 0.6^{a}	12.6 ± 0.6^{b}
Lipomyces tetrasporus Li-0407								
	SP-WSH 80	**SP-WSH 40**	**EP-WSH 80**	**EP-WSH 40**	**SP-WSH 80**	**SP-WSH 40**	**EP-WSH 80**	**EP-WSH 40**
Y DCW%	30 ± 2^{a}	26.4 ± 0.4^{b}	0.00 ± 0.00^{c}	25.0 ± 0.3^{b}	25 ± 2^{a}	26 ± 1^{a}	0.00 ± 0.00^{b}	28 ± 1^{a}
Prod	0.06 ± 0.01^{a}	0.05 ± 0.01^{ac}	0.00 ± 0.00^{b}	0.03 ± 0.01^{c}	0.05 ± 0.01^{a}	0.05 ± 0.01^{a}	0.00 ± 0.00^{b}	0.03 ± 0.01^{c}
Y lip%	17.5 ± 0.7^{a}	17.6 ± 0.8^{a}	0.00 ± 0.00^{b}	15 ± 2^{a}	15 ± 2^{a}	17.5 ± 0.9^{a}	0.01 ± 0.01^{b}	15 ± 1^{a}
Lip/DCW %	59 ± 9^{a}	67 ± 2^{a}	0.00 ± 0.00^{b}	63 ± 5^{a}	61 ± 3^{a}	66 ± 3^{ac}	0.01 ± 0.01^{b}	54 ± 3^{ad}

in the first 24 h in all the testes conditions. These molecules were not metabolised but were reduced into less toxic products, respectively 5-HMF alcohol and furfuryl alcohol by reductases and dehydrogenases in agreement with previous observations reported by Crigler et al., [58]. The greater sensitivity of EP cells compared to SP cells is well represented in Fig. 2.

Y. lipolytica inoculated in the SP phase, under all experimental conditions, obtained a biomass yield (Fig. 2 a, blue bar) and a lipid yield (Fig. 2 b, blue bar) higher than the EP inoculum (orange bar).

Fermentation tests with *Y. lipolytica* indicated that, even if the microorganism was able to operate a biological detoxification of WSH, the lipid yield was very low, with an average yield of 5% under all the studied conditions (Fig. 2b, blue and orange bar).

Lipid yields decreased by 50% compared to those obtained in synthetic medium. Probably, when the process was stopped, after 300 h, the yeast had not yet started the lipogenic phase, considering that about 50% and 60% of the total sugars (for WSH 40 and WSH 80, respectively) were still available in the medium (data not shown). Besides the intrinsic metabolic efficiency of the yeast, the flasks did not ensure adequate oxygenation of the medium or a regular pH control that could have limited the full uptake of the available carbon sources. In any case, the decrease in performance was most likely a consequence of the hydrolysate toxicity, which inhibited the lipids production by 50%, according with other studies [5,59].

Furthermore, as found in SYN medium, less than 10% mannitol was produced along with 3 unidentified compounds, likely belonging to the polyol groups or organic acid groups. Similar results were found by Giacomobono et al. [33], with *Yarrowia lipolytica* ATCC 46483. The authors reported mannitol and citric acid production using glycerol as the sole carbon source. Besides furan compounds, *Y. lipolytica* also metabolised acetic acid, which was consumed in the first 48 h. This is of great importance as short chain organic acids are among the most

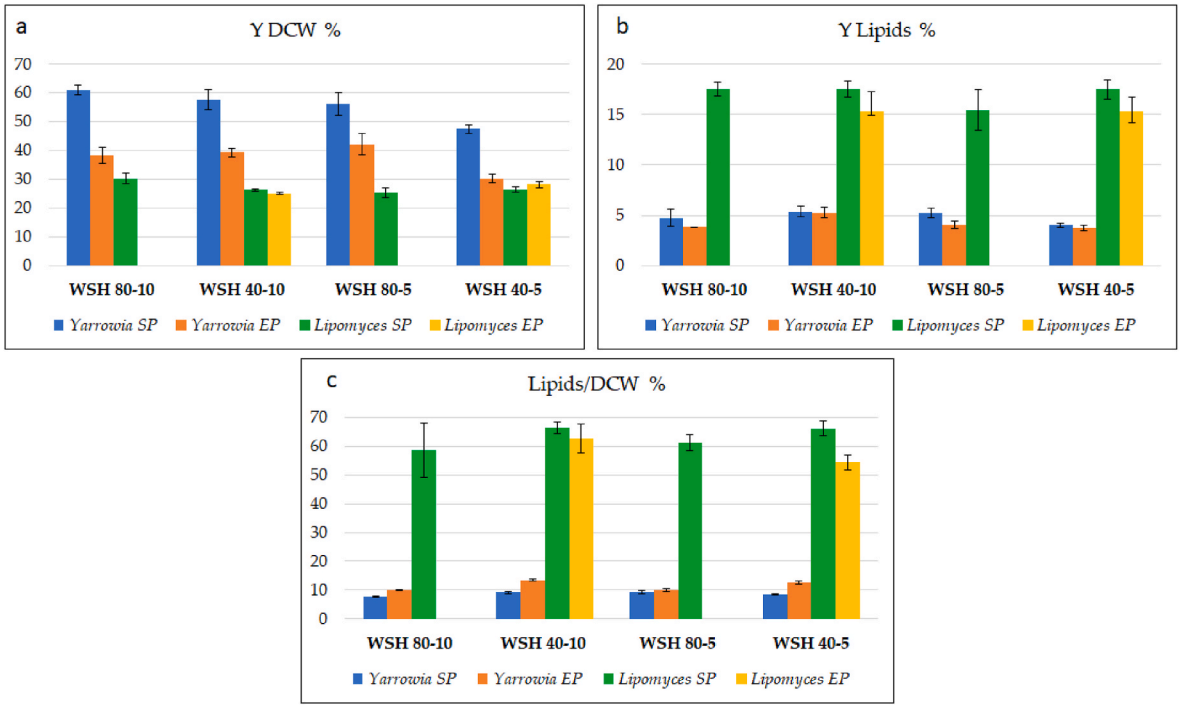

Fig. 2. *Production of biomass (Y DCW) and lipids (Y Lipids) as a function of the sugars consumed. Intracellular lipids accumulation (Lipids/DCW)calculated as lipids produced as a function of cell biomass.* Yarrowia lipolytica inoculated in Stationary Phase, SP (blue bar) and Exponential Phase, EP (orange bar), Lipomyces tetrasporus inoculated in Stationary Phase SP, (green bar) and Exponential Phase, EP (yellow bar). The concentration of the medium (WSH 80 or WSH 40) and the quantity of inoculum (OD 10 or 5) are reported on the abscissas.

abundant degradation by-products with inhibitory effects in biomass hydrolysates [60]. For example, Poontawee et al. [55], found a significant decrease in biomass and lipid concentration by 72% and 97%, respectively, for *R. fluviale* DMKU-SP314, when acetic acid concentration was higher than 1.0 gL^{-1}.

L. tetrasporus was never tested so far for the production of SCOs from undetoxified hydrolysates of wheat straw. The results in Table 4 show that the cells viability and metabolic capacity were severely affected by the hydrolysate composition ($p < 0.05$) The productivity was reduced by 67% compared to SYN tests. The growth phase of inoculum affected significantly the performance of this strain. In SP, *L. tetrasporus* was able to grow in any condition, differently in EP the yeast was able to grow only when inoculated in a medium with a low sugar concentration (Table 4). In these conditions, the process was very slow and the productivity was 3-folds lower than the SYN tests, and half that achieved with SP inoculum under the same conditions (Table 4). SP inoculum was able to metabolise furan compounds in the first 48 h and after that, the growth rate was comparable to the values observed in SYN medium. The EP inoculum, was 4-folds slower than SP inoculum and required around 200 h to detoxify the medium after that the growth proceeded as SYN medium (Supplementary). In high sugar medium, *L. tetrasporus* was unable to grow even after 200 h. Although the concentrations of inhibitors are the same in EP-WSH80 and EP-WSH40, the microorganism behaviour was extremely different on the two growth medium. This could be likely due to a higher osmotic stress in the WSH80 compared to WSH40. Similar effect of the sugars concentrations was previously described by Caporusso et al., [16].

Under the same conditions, the SP inoculum was very efficient and quick in detoxifying the hydrolyzate [16]. The different behaviour between EP and SP could lie in the stress tolerance mechanisms associated with different metabolic phases. In fact, it is well established from previous investigations on *Saccharomyces* strains that SP cells have a high tolerance towards multiple stress factors [61]. For example, the SP in *Saccharomyces cerevisiae* increases the robustness to thermal and osmotic shock [62] and increases the tolerance to acid compounds and wide pH range [17]. In this yeast, a thickening of the plasma membrane [63] and overexpression of alcohol dehydrogenase was also observed during SP [64]. Several studies have shown that these conditions play a crucial role in the stress tolerance of furan molecules as one of the enzymes responsible for the conversion of 5-HMF and furfural is aldehyde dehydrogenase [64]. Furthermore, numerous studies [65–68] observed a membrane damage due to furan molecules, consequently a membrane thickening could prevent or limit this effect. Similar tolerance mechanism might be correlated to the resistance of *L. tetrasporus* inoculated in SP.

Like *Y. lipolytica*, *L. tetrasporus* inoculated in the SP phase, obtained a biomass yield and a lipid yield (Fig. 2a and 2b, green bar) higher than the EP inoculum (Fig. 2a and 2b, yellow bar).

The maximum DCW yield, 30%, was obtained with SP cells (Fig. 2 a, green bar), grown on WSH 80 with inoculum density OD 10, whereas the maximum yield and lipid content, 17.6% (Fig. 2 b, green bar) and 67% (Fig. 2 c, green bar) respectively, were obtained with SP inoculum, WSH 40 and OD 10. A very similar result was obtained with the inoculum density corresponding to OD 5, namely 17% and 66% (Fig. 2b and c, green bar). It seems that the furan molecules did not have a negative effect on lipid synthesis; indeed, in some cases, higher yields were obtained on WSH (WSH 80, OD 10 and 5) compared to SYN. This result suggests that additional sources of carbon, present in WSH, were likely used by the yeast for growth and lipid production. Previous studies reported that *Rhodosporidium toruloides* achieved up to 30% more biomass, using WSH as a carbon source compared to SYN medium [38]. Probably, in the wheat straw hydrolysate, one of the non-sugar carbon sources can be represented by acetic acid. In all experimental conditions, acetic acid was no longer present in the medium at the end of the process.

From the analysis of the lipid profile no difference emerged between the growth on SYN medium and WSH. This result indicates the absence of correlation between the furan molecules or additional degradation products and the enzymes involved in the synthesis of FAs. This is in agreement with previous results which similarly demonstrated the absence of correlation with the lipid profile of *Rhodosporidium toruloides* [54] with furan compounds. On the other hand, it was observed that, besides the specific microorganisms, different carbon sources could determine different lipid profiles [69,70].

The data related to the composition of main fatty acids of lipids extracted by the two strains, after growth both in synthetic medium (SYN) and wheat straw hydrolysate (WSH) at different sugar concentration (80 and 40) and growth phase (SP and EP), were submitted to cluster analysis. The obtained dendrogram (Fig. 3) shows that the composition of lipid profile was mainly affected by the yeast strain. In fact, the fatty acids composition of lipids extracted by *L. tetrasporus* in all the tested conditions was included in the same group, which was separated by lipids extracted by *Y. lipolytica*.

The lipid profile of *Y. lipolytica* strains contained higher percentage of C18:2 and C16:1 (linoleic and palmitoleic acids, respectively) than lipids extracted by *L. tetrasporus*, which was characterized by higher percentage of C18:0 and C16:0 (stearic and palmitic acids, respectively). Each group can be further subdivided into two subgroups, in function of growth medium. For example, the lipid profile of *Y. lipolytica* strain, grown on synthetic medium was characterized by higher content of C18:0 than the profile obtained by this strain grown on WSH. In both the strains and in all the experimental conditions, oleic acid was the most abundant fatty acid.

3.4. Scale-up at 10 L bioreactor

On the basis of previous results, *L. tetrasporus* strain was selected for scaling-up the bioconversion process. The strain was cultured in a 10 L bioreactor in order to evaluate the kinetics of growth and lipid production on a medium-level scale. Since high glucose concentrations had a negative effect on yeast growth, a fed-batch process was implemented for sugar supplementation in order to avoid the negative effect induced by the high sugar concentration. The process was carried out using the conditions previously optimized. The obtained results are shown in Fig. 4. The initial sugars content was consumed after approximately 66 h of growth, whereas 5-HMF, furfural, and acetic acid were consumed in the first 24 h. Galactose was also metabolised in the first 24 h while the xylose uptake begins when the residual glucose concentration was comparable to xylose (15 gL^{-1}).

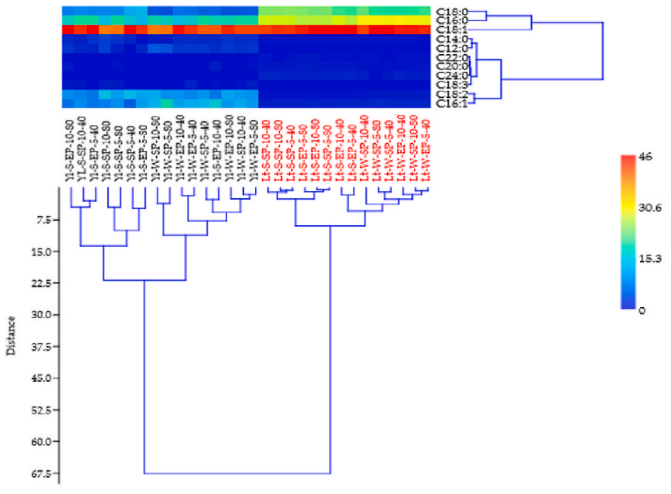

Fig. 3. Dendrogram obtained by cluster analysis on main fatty acids composition extracted by *Yarrowia lipolytica* (Yl, black) and *Lipomyces tetrasporus* (Lt, red) grown in synthetic medium (S) and wheat straw hydrolyzate (W), at low and high sugar concentrations (80 and 40), inoculated in Stationary (SP) and Exponential (EP) phase, at two inoculum levels (10 and 5 OD).

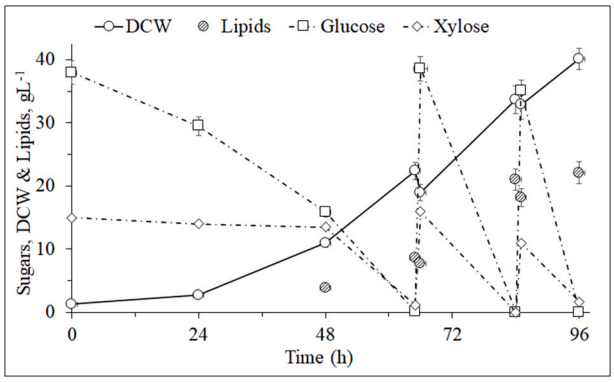

Fig. 4. Scale-up of the process at 10 L bioreactor, in fed-batch mode, with *Lipomyces tetrasporus* on WSH 40 inoculated in SP, with inoculum size of OD 5.

Compared with the flask tests, the bioreactor process was about 4-folds faster thanks to a controlled oxygenation combined with the regular pH adjustment. The maximum yield and lipid content of 21% and 62% respectively, were obtained at the end of the first feed (84 h) and, in these conditions, the maximum productivity of 0.25 $gL^{-1}h^{-1}$ was also achieved. At the end of the process, 40 gL^{-1} of DCW were obtained containing 23 gL^{-1} of lipids, accounting for 55% of the lipid content and 15.5% of lipid yield. These results indicated that a 25% decrease in the lipid yield was obtained after the second feed.

The lipid profile, summarized in Fig. 5, contained oleic acid (C18:1) 57%, palmitic acid (C16:0) 27%, stearic acid (C18:0) 6%, palmitoleic acid (C16:1) 5% and linoleic acid (C 18:2) 4%. Low quantities of lauric acid (C12:0), myristic acid, (C14:0) linolenic acid (C18:3), and arachidic acid (C20:0) were also found. Interestingly, oleic acid was increased by 27% compared to the tests in the flasks, whereas the stearic acid production decreased by 20%. Probably, the higher dissolved oxygen in the bioreactor condition increased the unsaturation level of the fatty acids [71]. The fatty acids profile achieved in the present paper was similar to that previously reported by ourselves for the growth of *L. tetrasporous* on cardoon hydrolysates having a similar sugars composition. These results confirm that, even if the lipids yields could be affected by the specific hydrolysates composition, the fatty acids profile depend only on the microorganism species and process conditions.

3.5. Synthesis and characterization of biodiesel

One potential application of microbial oils is the biodiesel production. The extracted lipids were submitted to the transesterification process reaching about 85% conversion rate. To investigate the properties of the obtained biodiesel (Table 5) four physical properties were measured: water content, density (ρ), kinematic viscosity at 40 °C (ν),

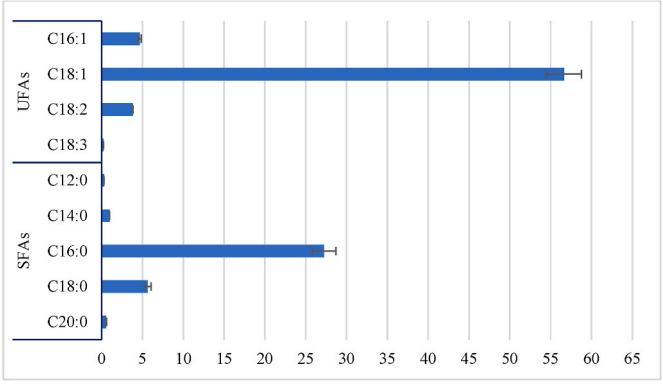

Fig. 5. Fatty acids profile *of Lipomyces tetrasporus* grown in 10 L bioreactor and fed-batch mode. UFAs = Unsaturated fatty acids; SFAs = Saturated fatty acids.

Table 5
Biodiesel properties obtained from *L. tetrasporus* lipids, compared with vegetable oils (palm oil and rapeseed oil) and with international biodiesel standard, US biodiesel ASTM D6751 and EU biodiesel EN 14214. Standard deviations were of the order of 4%. ns > not specified.

	Density ρ (g/cm^3)	Viscosity ν (mm^2/sec)	HCV (MJ/Kg)	IV (mgI_2/100 g)	CN	CFPP (C°)
L. tetrasporus	0.89	3.97	37.60	60.50	60.90	6.50
Palm oil	0.87	4.61	40.60	54.00	61.90	9.00
Soybean oil	0.88	4.26	39.70	125.50	51.30	−4.00
ASTM D6751	*ns*	1.90–6.00	*Ns*	*ns*	≥47	*ns*
EN 14214	0.86–0.90	3.50–5.00	*Ns*	≤120	≥51	*ns*

and calorific value (High Calorific Value (HCV), and Low Calorific Value, (LCV)). Furthermore, through the empirical formulas reported in Viola et al. [27], the values of Cetane Number (CN), Iodine Value (IV), and Cold-Filter Plugging Point (CFPP) were calculated. The biodiesel characteristics were then compared with vegetable oils (palm oil, and soybean oil [72]) and international biodiesel standards required to use biodiesel for diesel engines, namely ASTM D6751 (United States) and EN 14214 (European Union). As shown in Table 5, most properties of the microbial biodiesel are similar to those obtained from vegetable oils. For biodiesel purification, hot water is usually used for washing, in order to remove the polar fraction. Likewise, traces of water can pollute biodiesel, so it must be removed. The residual water content in the microbial biodiesel was 450 ppm, below the threshold value of 500 ppm (EN 14214 and ASTM D6751). The ρ value was very similar among the 3 analysed oils, although the density of the microbial biodiesel was the highest, 0.892 g/cm^3. Density plays a crucial role to determine the fuel injection property as it affects the pumping of fuel by its volume [73]. It was reported that denser biodiesel has more energy than petroleum diesel [74]. Density is limited to 0.860–0.900 g/cm^3 at 15 °C in EN 14214 but there is no specification for density in the ASTM D6751.

Kinematic viscosity (ν) is a property of the fuel that defines the flow capacity, speed, and quality of the spray injected into the combustion chamber of the engine. The high viscosity of biodiesel causes large droplets and poor vaporization. This leads to scarce combustion and therefore to an increase in emissions [75]. The viscosity increases with the length of the fatty acid chain or with the unsaturation of the fatty acid and depends on the number and nature of the double bonds. The ν of the microbial biodiesel was 3.9 mm^2/s, a value lower than ν reported for vegetable oils and in the ranges defined by US and EU standards, 1.9–6.0 mm^2/s and 3.5–5.0 mm^2/s, respectively.

High Calorific Value (HCV) characterizes the energy content of fuels and thereby their efficiency. The HCV increases with the increasing chain length of methyl esters and with the ratio of carbon and hydrogen to nitrogen and oxygen increase [76]. Both EU and US biodiesel standards do not have any specification for HCV value. However, a low HCV value of microbial biodiesel (37.6 MJ/kg) will result in higher fuel consumption to achieve a similar yield of biodiesel from soybean or palm oil. Furthermore, from the HCV it was calculated the LCV value on the basis of the elemental composition of biodiesel. The LCV value indicates the amount of energy that could be transferred during combustion. The LCV obtained was 35.23 MJ/kg.

The Iodine Value (IV) refers to the tendency of the biodiesel to react with oxygen at room temperature. High IV causes the polymerization of glycerides, and rubber formation by heating. It depends on both the number and position of the double bonds in the FAMEs. Unsaturated fatty acids methyl esters crystallize more slowly than saturated fatty acids, therefore, biodiesel with high levels of unsaturated fatty acid methyl esters is more suitable for cold countries [77]. On the other hand, unsaturated compounds can reduce the oxidation stability of biodiesel because double bonds are susceptible to being oxidized. EU has set a limit of IV at 120 g I_2/100 g, which excludes soybean oils as feedstocks

for biodiesel production. In this study, about 65% of microbial oils was composed of unsaturated fatty acids methyl esters, and the IV of microbial biodiesel was 60.5 mgI_2/100 g.

The Cetane Number (CN) provides information on the combustion behaviour, in particular on the ignition time delay in the combustion chamber. A low CN is related to inefficient combustion, and therefore resulting in increase of exhaust gases and particulate emissions [78]. In this study, the CN of microbial biodiesel is 60.9, very similar to palm oil (61.9) biodiesel and 17% higher than soybean oil (51.3) biodiesel. Furthermore, the CN value meets the value of EU (≥51) and US (≥47) quality standards.

To determine the behaviour of biodiesel at low temperatures it is necessary to calculate the Cold Filter Plugging Point (CFPP). At low temperatures the saturated fatty acids C16: 0 and C18: 0 precipitate, forming solids that clog the fuel filter or block the engine [79]. No specific recommendation in the European standard based on this value is available. In this study, the value was quite high, 6.5 °C, but it can be improved by blending the biodiesel with alcohol, such as butanol or methanol [80].

4. Conclusions

The present study reported a detailed investigation of *Lipomyces tetrasporus* Li-0407 and *Yarrowia lipolytica* ATCC 46483 grown for the first time on undetoxified hydrolysates of steam pretreated wheat straw for the production of SCOs and advanced biodiesel. The process was extensively investigated at flask scale by exploring the effect of the inoculum age, cell density and medium composition. The results indicated that *Y. lipolytica* grew in undetoxified hydrolysates, reaching biomass yields of 61% but achieved lower lipids yields of 5% compared to *L. tetrasporus*. The inoculum age played a decisive role in determining a sharp difference in the cell response to microbial stress. In the stationary phase of the growth, *L. tetrasporus* inoculum showed an enhanced tolerance to the stress condition, compared to the exponential phase. Furthermore, higher growth and lipid production was documented in medium with low sugar concentration. Under these conditions, namely glucose and xylose concentration of 40 and 22 gL^{-1}, the lipid yield and cell accumulation were 18% and 67% respectively. The optimized process conditions for the conversion of undetoxified wheat straw hydrolyzates by *L. tetrasporus* were finally tested in a medium scale bioreactor (10 L bioreactor), using a fed-batch process enabling a final lipid yield and content of 21% and 62%, respectively. The *L. tetrasporus* oil was extracted and transesterified to achieve biodiesel whose chemical-physical properties resulted very similar to palm or soy oils and could, therefore, represent a potential substitute for vegetable oils in the production of advanced biodiesel and additional oil-based advanced biofuels.

CRediT authorship contribution statement

Antonio Caporusso: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Validation, Writing – original draft, Writing – review & editing. **Isabella De Bari:** Conceptualization, Project administration, Supervision, Writing – review & editing. **Federico Liuzzi:** Data curation, Investigation, Methodology, Writing – original draft. **Roberto Albergo:** Investigation, Writing – original draft. **Vito Valerio:** Investigation, Methodology, Validation. **Egidio Viola:** Validation, Writing – original draft. **Rocchina Pietrafesa:** Data curation, Formal analysis, Software, Writing – original draft. **Gabriella Siesto:** Data curation, Formal analysis, Software, Writing – original draft. **Angela Capece:** Conceptualization, Project administration, Software, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.renene.2022.11.059.

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