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Lipid Production by Yeasts Growing on Commercial Xylose in Submerged Cultures with Process Water Being Partially Replaced by Olive Mill Wastewaters

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Abstract: Six yeast strains belonging to *Rhodosporidium toruloides*, *Lipomyces starkeyi*, *Rhodotorula glutinis* and *Cryptococcus curvatus* were shake-flask cultured on xylose (initial sugar—S_0 = 70 ± 10 g/L) under nitrogen-limited conditions. *C. curvatus* ATCC 20509 and *L. starkeyi* DSM 70296 were further cultured in media where process waters were partially replaced by the phenol-containing olive mill wastewaters (OMWs). In flasks with S_0 ≈ 100 g/L and OMWs added yielding to initial phenolic compounds concentration (PCC_0) between 0.0 g/L (blank experiment) and 2.0 g/L, *C. curvatus* presented maximum total dry cell weight—$TDCW_{max}$ ≈ 27 g/L, in all cases. The more the PCC_0 increased, the fewer lipids were produced. In OMW-enriched media with PCC_0 ≈ 1.2 g/L, TDCW = 20.9 g/L containing ≈ 40% *w/w* of lipids was recorded. In *L. starkeyi* cultures, when PCC_0 ≈ 2.0 g/L, TDCW ≈ 25 g/L was synthesized, whereas lipids in TDCW = 24–28% *w/w*, similar to the experiments without OMWs, were recorded. Non-negligible dephenolization and species-dependent decolorization of the wastewater occurred. A batch-bioreactor trial by *C. curvatus* only with xylose (S_0 ≈ 110 g/L) was performed and TDCW = 35.1 g/L (lipids in TDCW = 44.3% *w/w*) was produced. Yeast total lipids were composed of oleic and palmitic and to lesser extent linoleic and stearic acids. *C. curvatus* lipids were mainly composed of nonpolar fractions (i.e., triacylglycerols).

Keywords: lignocellulosic sugars; microbial lipid; olive mill wastewater; *Cryptococcus curvatus*; *Lipomyces starkeyi*

1. Introduction

Lignocellulosic materials represent the largest and the most attractive biomass resource worldwide that can serve as cheap feedstock of monosaccharides in a remarkable plethora of microbial fermentations. These low-cost materials like woody biomass, grass, agricultural and forestry solid wastes, process waters rich (or potentially very rich) in lignocellulosic sugars (i.e., spent sulfite liquor, waste xylose mother liquid), municipal solid wastes, etc., are particularly abundant in nature and have a very important potential for various types of microbial bioconversions [1–8]. Commercial xylose, being one of the principal monomer sugars of these feedstocks, is produced after acid hydrolysis of various types of lignocellulosic biomass (i.e., corn cobs, sugarcane bagasse, etc.) [2,3,7], followed by subsequent condensation and crystallization [1,6]. The capability of microorganisms to grow on and produce biotechnological compounds during culture on C-5 sugars and specifically during

fermentations on xylose, that, as stressed, is one of the most abundant sugars on the lignocellulosic biomass, presents continuously growing importance [3–5,7,8].

Olive mill wastewaters (OMWs) are the major effluent deriving from olive oil production process, specifically when traditional (viz. press extraction systems) or 3.0- and 2.5-phase centrifugation systems are employed. Despite the gradual utilization of 2.0-phase centrifugation systems (that generate lower OMW quantities than the 3.0- or the 2.5-phase systems) within the EU countries, OMWs are always considered as one of the most important agro-industrial wastes generated into the Mediterranean region; these residues are seasonally produced in very high quantities, whereas their strong odor and dark color as also their relatively high organic load have a direct negative impact on the environment if they are released without previous treatment. This important residue of the olive oil industry is one of the most difficult to treat wastes because of its high content in phenolic compounds [9,10]. The increased concentration of OMWs in organic matter and phenolic components results in reduction of the available concentration of oxygen when these residues are released without prior treatment and this upsets the balance of ecosystems and the soil porosity, resulting in contaminated aquifers and polluted environments [10–12]. In addition, the phenolic compounds found into the OMWs are, in general, quite instable and their polymerization during uncontrolled release often leads to the generation of high-molecular-weight, hardly degradable substances [10,13,14]. The annual production of OMWs is estimated to be $> 15 \times 10^6$ m^3 [13] and this very high residue production together with the seasonal production of these wastewaters render their environmentally friendly disposal and management as very important priorities, specifically for the Mediterranean countries [9,13,14].

The last years a new trend has appeared in relation to the valorization various types of food-processing wastewaters or low-quality waters referring to their simultaneous utilization as substrate and as fermentation water implemented in various types of microbial-guided bioprocesses. Specifically, "concentrated" wastes and residues (like crude glycerol, molasses, etc.) and renewable low- or zero-cost hydrophilic (i.e., glucose syrups, low-purity sugars, white grape pomace, etc.) or hydrophobic (i.e., various low quality fatty compounds) carbon sources could be diluted; in this type of dilution, instead of the tap water, OMWs (or similar types of wastewaters like the table olive-processing wastewaters) can be used as process waters. Accordingly, added-value metabolites could be produced during these fermentation processes with simultaneous partial detoxification (i.e., decolorization and phenol removal) of the implicated wastewaters [11,12,15–21]. On the other hand—and in combination with the strategy previously mentioned—OMWs could initially be subjected to purification in order to recover useful materials that are found into the residue (i.e., antioxidant compounds) [14], and, thereafter, utilize the bulky remaining wastewater as process water and simultaneous substrate in fermentation processes.

Microbial lipids (single-cell oils—SCOs) are produced by the "oleaginous" microorganisms (these that can accumulate lipid to quantities ≥20% in DCW during growth on glucose in conditions favoring lipogenesis; [22,23]). These lipids possess similar fatty acid (FA) composition with various edible oils [24]. Therefore, these fatty materials can be implicated as perfect agents in the synthesis of the so-called "2nd generation" biodiesel (in fact, it is the biodiesel the production of which is not in competition with the arable land) [3,5,22,25]. SCOs can also be employed as starting materials for the synthesis of several added-value oleochemical compounds or can be implicated as high added-value fatty supplements in the food-processing industries (specifically the ones that contain rarely found poly-unsaturated FAs or these that have composition similarities with expensive exotic fats) [3–5,7,8,22,23,26,27].

In the current investigation, a number of yeasts belonging to the species *Rhodosporidium toruloides*, *Rhodotorula glutinis*, *Cryptococcus curvatus* and *Lipomyces starkeyi* (in total six strains) were tested as regards their potential to assimilate commercial, non-purified xylose, deriving from lignocellulosic biomass. Trials were carried out in shake-flask mode under nitrogen-limited conditions, favoring the production of SCO [22–24]. In the next step, the most promising among the previously screened yeasts, namely *Cryptococcus curvatus* ATCC 20509 and *Lipomyces starkeyi* DSM 70296, were cultivated in

media presenting high initial xylose concentrations with initial nitrogen remaining constant, in order to further enhance the production of SCO. Moreover, according to a new trend recently appeared in the Industrial Biotechnology, OMWs were employed as liquid medium in order to partially replace tap water in the fermentation carried out. Finally, *C. curvatus* ATCC 20509 culture that presented very interesting total biomass and SCO production on high-xylose concentration media was scaled-up in bench top laboratory-scale bioreactor. Quantitative and kinetic considerations concerning the yeast physiological behavior were critically discussed.

2. Materials and Methods

2.1. Microorganism, Media and Cultures

The strains used in the present study belonged to the species *Rhodosporidium toruloides* (strains DSM 4444 and NRRL Y-27012), to the species *Rhodotorula glutinis* (strain NRRL YB-252), to the species *Cryptococcus curvatus* (strains ATCC 20509 and NRRL Y-1511) and to the species *Lipomyces starkeyi* (strain DSM 70296). The strain with the code nomination ATCC was given from the American Type Culture Collection, the strains with the code nomination DSM were provided by the German Collection of Microorganisms and Cell Cultures, while strains encoded NRRL Y-derive from the NRRL culture collection. With the exception of *R. toruloides* and *R. glutinis*, strains were maintained on YPDA slants (10-g/L glucose, 10-g/L yeast extract, 10-g/L peptone and 20-g/L agar) at 4 °C. *R. toruloides* and *R. glutinis* were maintained on YPDMA medium (10-g/L glucose, 10-g/L yeast extract, 10-g/L malt extract, 5-g/L peptone and 20-g/L agar) at 4 °C.

Most of the experiments were carried out in 250-mL Erlenmeyer flasks containing 50 ± 1 mL of growth medium, previously sterilized at T = 115 ± 1 °C for 20 min and inoculated with 1 mL of 24-h exponential preculture yeast incubated at 180 rpm at T = 28 ± 1 °C (use of an orbital shaker Zhicheng ZHWY 211C; PR of China). This type of shake-flask culture performed in the current investigation (utilization of 250-mL flasks filled with the ⅕ of their volume and agitated at 180 rpm) implicates full aerobic conditions (viz. dissolved oxygen tension—DOT ≥ 20% *v*/*v*) throughout the flask experiments performed [11,12,16]. The yeast preculture was carried out in YPD medium. Liquid cultures were performed in a medium in which the salt composition was as in Papanikolaou et al. [28]. Yeast extract at 3.0 g/L and peptone at 1.0 g/L were used as nitrogen sources. Yeast extract contained *c.* 7% *w*/*w* nitrogen, while peptone contained *c.* 14% *w*/*w* nitrogen, respectively. Commercial-type xylose (purity of *c.* 95%, *w*/*w*) implicated as starting material for the large-scale synthesis of xylitol, was used as the sole carbon source. In the first part of the study, a screening of all available microorganisms was carried out on media containing xylose at initial total sugar (S_0) concentration ≈70 g/L. In the nest step of the experimental procedure and in order to further enhance the production of cellular lipids, the most promising of the previously screened microorganisms (it was found that were mainly the strains *C. curvatus* ATCC 20509 and *L. starkeyi* DSM 70296) were cultured on media containing higher S_0 concentration (≈100 g/L) with initial nitrogen concentration into the medium remaining the same as in the previously mentioned screening experiment (yeast extract at 3.0 g/L and peptone at 1.0 g/L), therefore trials in media with higher initial C/N molar ratio were prepared.

In one case (trial using xylose at S_0 ≈ 100 g/L, with initial nitrogen concentration as mentioned above), a batch bioreactor trial was performed by the strain *C. curvatus* ATCC 20509. The culture was carried out in a bench top 3-L bioreactor (New Brunswick, SC, USA), containing 1.9 L of fermentation medium. The reactor was aseptically inoculated with 100 mL of 24-h yeast preculture (see details in the previous section). The culture conditions in the bioreactor trials were as follows: incubation temperature 28 ± 1 °C; agitation rate 450 ± 5 rpm, aeration 0.1–1.5 vvm (the aeration was set on cascade mode, and the air-pump debit varied automatically within the above-mentioned vvm values in order to maintain a dissolved oxygen tension (DOT) value above 20%, *v*/*v*, in all culture phases); pH = 6.0 ± 0.1 regulated with automatic addition of KOH (2 M).

In the frame of sustainability and water-saving policies adopted, fermentation waters were partially replaced by olive mill wastewaters (OMWs) obtained from a three-phase decanter olive mill in the region Kalentzi (Corinthia, Peloponnisos, Greece). After their collection, OMWs were immediately frozen at–20 ± 1 °C and before use, these wastes were thawed and the solids were removed by centrifugation and subsequent filtration [17], to ensure the uniformity of the liquid material implicated in the fermentations. This procedure is in accordance with the current Greek legislation [29] regarding pretreatment of the waste before its disposal. Before fermentations, chemical analyses of the used OMWs that would replace process water were carried out; the initial pH of OMWs used was = 4.9 ± 0.1. Initial phenolic compounds concentration (PCC_0) in the residue was found to be ≈3.5 g/L (phenolic compounds (PC) were expressed as gallic acid equivalents). Moreover, OMWs contained a small concentration of total sugars (≈10.0 g/L). These sugars, after HPLC analysis conducted (see analysis details in the next paragraphs) were mainly composed of glucose (*c.* 80% of sugars) and fructose (*c.* 20% of sugars). Insignificant quantities of organic acids (citric acid at *c.* 2.0 g/L and acetic acid at *c.* 2.5 g/L—analysis equally performed through HPLC) were also detected. Protein quantity into the residue, as quantified through the Lowry method, was found to be ≈3.0 g/L. The quantity of ammonium ions, as determined with the aid of an ammonium selective electrode (SA 720, Orion, Boston, MA, USA) was found to be ≈10 mg/L. The electrical conductivity of the wastewater at 25 °C was 13.85 ms/cm (Hanna HI-8733 conductivity meter, Padova, Italy). Finally, triple extraction of OMWs by hexane revealed very small concentrations of fatty materials (0.4 ± 0.1 g/L of olive oil) in the volume of the residue. In the cases in which OMWs partially replaced the process fermentation waters in the cultures carried out, due to the indeed low concentrations of proteins, organic acids and oils, these compounds were not taken into consideration concerning their impact upon the quantitative physiological response of the microorganisms tested. In contrast, in the quantitative determination of total sugars in the growth medium, the contribution of sugars added from the OMWs, although almost negligible as compared with the initial concentration of xylose added, was taken into consideration.

The initial pH in the culture media after the addition of the salts was found to be = 6.0 ± 0.1 while pH value during all trials was always maintained between the ranges of 5.2–5.8, due to the buffer capacity of the salts of the employed medium. Since microorganisms did not produce appreciable quantities of organic acids, no external addition of base was requested in order to maintain the pH value within this range.

2.2. Determinations and Analyses

For the flask cultures, flasks were periodically removed from the incubator while for the bioreactor experiments *c.* 10 mL of culture was aseptically collected. In the flask trials, pH was measured in a SevenCompact™ pH S210 pH meter (Mettler-Toledo, Columbus, OH, USA). The whole content of the 250-mL flasks or the sample collected from the bioreactor experiment were subjected to centrifugation at 9000× *g*/10 min at 4 °C (Universal 320R-Hettich centrifuge, Tuttlingen, Germany). Wet biomass collected after the first centrifugation, was then extensively washed with distilled water and centrifugation was applied once more at the same conditions. Wet yeast biomass was dried at T = 90 ± 5 °C for 24 h to obtain the total dry cell weight (TDCW—*X*, in g/L) [28]. Biomass yield ($Y_{X/S}$, in g/g), was expressed as the grams of cell dry weight (*X*) produced, per grams of total sugar (mostly or completely xylose; *S*) consumed (g TDCW/g total sugars consumed).

Total cellular lipid (*L*) extracted from the TDCW with a chloroform-methanol mixture (2/1, *v*/*v*) according to Gardeli et al. [30], was determined gravimetrically and was expressed as g/L. Lipid in TDCW (%, *w*/*w*; $Y_{L/X}$), was calculated based on percentage of the accumulated lipid (*L*, g/L) per produced TDCW (*X*, g/L). Extracted intracellular lipids were converted to their fatty acid methyl esters (FAMEs) and analyzed in a gas chromatography (GC) according to Fakas et al. [31]. Specifically, a quantity of total lipids (<100 mg) was converted to the respective methyl esters in a two-step reaction using $CH_3O^-Na^+$ and CH_3OH/HCl as methylation agents [31]. The identification of the analyzed methyl esters was based on the comparison of retention times with known FAMEs standards. In the

representations done, the relative area of the FAMEs is presented. Moreover, in some instances, crude lipid of *C. curvatus* ATCC 20509 was washed with 0.88%-*w*/*v* KCl solution and was subsequently fractionated to its principal lipid fractions (viz. neutral lipids (NLs), glycolipids plus sphingolipids (G + S) and phospholipids (PLs), respectively) according to Fakas et al. [31] and Gardeli et al. [30]. Lipid fractions were trans-methylated according to the previously mentioned protocol. Thin layer chromatography (TLC) analysis in this crude lipid ("Folch" extract) was performed on glass pre-coated silica gel G plates (Merck, Darmstadt, Germany) (20 cm × 20 cm; thickness = 0.25 mm) as previously described [30]. Plates were pre-washed and activated according to Papanikolaou et al. [32] and separation of crude total lipid was carried out with petroleum ether/diethyl ether/glacial acetic acid (80:20:1, *v*/*v*/*v*). Plate application of the sample and the lipid standards and visualization after the development were conducted according to Papanikolaou et al. [32]. Glyceryl trioleate (TAG), cholesterol (CL), cholesteryl linoleate (CE), oleic acid (FFA), and monononadecanoin (MAG) [33] were employed as lipid standards for the identification of the main bands on TLC plates.

Determination of total intracellular polysaccharides (IPS) was carried out using a modified protocol described by Diamantopoulou et al. [34]. In brief, a precisely weighted quantity of TDCW (≤100 mg) was subjected to boiling (100 °C) with 20 mL of 2.5-N HCl for 1 h, and afterwards the whole was neutralized to pH = 7.0 with 2.5-N NaOH. Then, IPS were quantified as glucose equivalents with the DNS method [35] and were expressed in both absolute (g/L) and relative ($Y_{IPS/X}$, % of total polysaccharides in TDCW) values.

Total reducing sugars in the fermentation medium (viz. xylose for the trials with no OMWs, mostly xylose and to lesser extent glucose and fructose for the trials in which OMWs were added) were quantified according to the DNS method [35]. Pure xylose (purity = 99% *w*/*w*) was employed in order to trace the calibration curve. In some instances, and in order to perform cross-validation of the results, the concentration of the remaining xylose was also determined during the fermentation using HPLC. Xylitol, produced in small concentrations and organic acids and sugars found in the OMWs, were also determined through HPLC analysis, performed according to Papanikolaou et al. [16]. The area of each compound was determined according its retention time and the concentration of each compound was determined using reference curves and expressed as g/L. Free amino nitrogen (FAN) concentration into the liquid samples was determined at 570 nm using the ninhydrin colorimetric method with glycine employed in order to trace the calibration curve, according to Kachrimanidou et al. [36].

Determination of total phenol compounds concentration (PCC) into the medium was carried out according to the method described by the Folin–Ciocâlteu protocol modified according to Aggelis et al. [37] in the sample supernatant. Absorbencies were measured at 750 nm in a Hitachi *U*-2000 Spectrophotometer (Hitachi High Technologies Corp., Fukuoka, Japan). The concentration of phenolic compounds was expressed in equivalence of gallic acid according to a reference curve. Moreover, in order to determine the decolorization efficiency of the fermentations, 0.5-mL samples were mixed well with 14.5 mL distilled water and the absorbance was measured at 395 nm (same spectrophotometer as previously) according to Sayadi and Ellouz [38]. The decolorization percentage was calculated using the equation: % A = $[(A_0 - A_1)/A_0] \times 100$, where, A_0 is the absorbance at time 0 and A_1 is the absorbance at each experimental point during the fermentation.

2.3. Data Analysis

Each experimental point of all the kinetics presented in tables and figures is the mean value of two independent determinations; in fact, for each experiment presented, two lots of independent cultures using different inocula were conducted; the standard error (SE) was < 15%. Data were plotted using Kaleidagraph 4.0 Version 2005 showing the mean values with the standard error mean. Throughout the text, indices 0 and max represent the initial and the maximum quantity of the elements in each kinetics presented.

3. Results and Discussion

3.1. Initial Screening on Commercial-Type Xylose

In the first part of this investigation, all available strains were cultivated on commercial-type xylose at initial total sugar (xylose) (S_0) concentration ≈70 g/L under nitrogen-limited conditions. The achieved kinetic results are illustrated in Table 1. All strains presented appreciable TDCW production (X_{max} ranging between 15.6 and 19.0 g/L, respective total biomass yield on sugar consumed $Y_{X/S}$ ≈ 0.22–0.28 g/g). $Y_{X/S}$ values seemed to be slightly higher in the middle of the cultures and before X_{max} values were recorded. Moreover, variable quantities of SCO were produced by the microorganisms tested; the lower lipid in TDCW quantities ($Y_{L/X}$, % *w*/*w*) were recorded for the strains *Rhodosporidium toruloides* NRRL Y-27012 ($Y_{L/X}$ < 20% *w*/*w*) and *Rhodotorula glutinis* NRRL YB-252 ($Y_{L/X}$ ≈ 10% *w*/*w*) despite the fact that growth was conducted in media favoring the production of SCO. This result is in disagreement with recent investigations demonstrating the strain *R. toruloides* NRRL Y-27012 produced huge quantities of SCO ($Y_{L/X}$ > 40% *w*/*w*, in some trials this value was = 54.3% *w*/*w*, with an L_{max} value ≈ 12 g/L) on media composed of crude glycerol, the principal waste stream deriving from biodiesel production process [39]. Equally, significant SCO quantities were produced by the strain *R. glutinis* NRRL YB-252 ($Y_{L/X}$ = 38.2% *w*/*w*, corresponding to L_{max} ≈ 7.2 g/L) in similar types of media (crude glycerol) [40], demonstrating that in the above-mentioned yeast, as well as in a plethora of other yeast species and genera, glycerol is a very competitive substrate related with the production of TDCW and added-value extracellular and intracellular metabolites [33,40–43]. It is not clearly understandable why such important discrepancies exist between the lipid production process for *R. toruloides* NRRL Y-27012 and *R. glutinis* NRRL YB-252 growing on xylose and glycerol, but in any case—and in accordance with the literature [4,5,22,23,44,45]—it appears that the carbon source seems to play a very crucial role in the de novo lipid production process, even if implicated substrates present important similarities among them in molecular and metabolic level. In this section, though, it must be indicated that while the intracellular metabolism of glycerol and glucose (or other hexoses) theoretically presents important similarities (these compounds are mostly metabolized through the EMP pathway) [5,22,23], the intracellular metabolism of xylose could present some differences compared with the above-mentioned compounds. These differences could, finally, reflect in the quantity of cellular lipids produced by the microorganisms growing on these substrates; for instance, xylose metabolism implicates in many instances the pentose–phosphate pathway [5,22,23], that is somehow less efficient as regards biomass production compared with the typical EMP glycolysis utilized for the catabolism of hexoses or glycerol [5,22]. On the other hand, xylose metabolism implicates in its first step, the reduction of xylose into xylitol, that in many cases in which oleaginous microorganisms are involved [30,33] is secreted into the medium and it is not re-utilized in order to be converted into SCO. Therefore, there would be a carbon "loss" related with the production of microbial lipids when xylose is used as microbial substrate amenable to be converted into cellular lipid compared with utilization of hexoses or glycerol [22,30,33].

The remaining "red" yeast strain implicated in the current investigation, namely *R. toruloides* DSM 4444, presented efficient cell growth and non-negligible lipid accumulation ($Y_{L/X}$ ≈ 30% *w*/*w*) on media composed of commercial-type xylose (Table 1). The above-mentioned strain has been revealed capable to produce interesting SCO quantities during growth of glycerol and, to lesser extent glycerol/xylose blends [33], whereas it accumulated indeed impressive quantities of storage lipid during growth on flour-rich waste stream hydrolysates, mostly composed of glucose, during growth in shake-flask or bioreactor experiments [46].

C. curvatus NRRL Y-1511 presented the highest TDCW production among all tested strains cultivated on commercial-type xylose (X_{max} = 19 g/L) even though this strain did not produce significant SCO production under the given culture conditions ($Y_{L/Xmax}$ < 25% *w*/*w* for all growth steps) (Table 1). The very same has been reported to produce a SCO quantity = 4.3 g/L (respective $Y_{L/Xmax}$ value ≈ 30% *w*/*w*) during growth on lactose, in shake-flask fermentations [47]. Harde et al. [48] reported a SCO

produced quantity = 5.1 g/L (respective $Y_{L/X} \approx 38\%$ *w*/*w*) during growth of the above-mentioned yeast strain on xylose-based shake-flask fermentations. On the other hand, the same strain was not capable to produce significant SCO quantities ($Y_{L/X}$ values always < 20% *w*/*w*) during growth on media composed of saccharose (i.e., molasses, crude sucrose, orange peel waste extracts) [47,49]. Moreover, in the current submission, *L. starkeyi* DSM 70296 was proved to be a robust TDCW-producing strain during growth on commercial-type xylose, in shake-flask experiments under nitrogen-limited conditions, although total lipid quantities produced ($Y_{L/Xmax} \approx 34\%$ *w*/*w*) were somewhat or remarkably lower compared with the ones achieved on glycerol [39] or (mostly glucose-based) flour-rich waste stream hydrolysates [50] in shake-flask and bioreactor experiments. All these results and their comparisons with the literature indicate, once more, that important differentiations in the lipid production bioprocesses may exist even when carbon sources presenting biochemical similarities are used as substrates for the given oleaginous microorganisms employed.

Table 1. Quantitative data of yeast strains deriving from experiments performed on commercial-type xylose, in nitrogen-limited shake-flask cultures, in which the initial sugar concentration was adjusted to *c.* 70 g/L. Four different points in the fermentations are represented: (a) when the maximum quantity of total dry cell weight (TDCW—*X*, g/L) was observed; (b) when the maximum quantity of total cellular lipids (*L*, g/L) was observed; (c) when the maximum quantity of xylitol (Xyl, g/L) was observed; (d) when the maximum quantity of total intracellular polysaccharides (IPS, g/L) was observed. Fermentation time (h), quantities of TDCW (*X*, g/L), total lipid (*L*, g/L), intracellular polysaccharides (IPS, g/L), total sugar (xylose) consumed (S_{cons}, g/L), lipid in TDCW ($Y_{L/X}$,% *w*/*w*) and polysaccharides in DCW ($Y_{IPS/X}$,% *w*/*w*) are depicted for all the above-mentioned fermentation points. Culture conditions: growth on 250-mL conical flasks filled with the ⅕ of their volume at 180 ± 5 rpm, initial pH = 6.0 ± 0.1, pH ranging between 5.2 and 5.8, incubation temperature 28 °C. Each experimental point is the mean value of two independent measurements (SE < 15%).

Yeasts		Time (h)	S_{cons} (g/L)	*X* (g/L)	*L* (g/L)	Xyl (g/L)	$Y_{L/X}$ (%, *w*/*w*)	IPS (g/L)	$Y_{IPS/X}$ (%, *w*/*w*)
Rhodosporidium	c	72	26.8	5.6	0.6	4.3	10.7	1.3	23.2
toruloides	d	120	37.2	10.7	2.8	4.0	26.2	2.3	21.4
DSM 4444	a, b	264	57.8	16.5	4.8	3.1	29.1	1.7	10.3
Rhodosporidium	d	120	40.1	11.9	1.3	Tr.*	10.9	3.2	26.9
Toruloides NRRL Y-27012	a, b, c	264	59.0	15.6	3.0	Tr.*	19.5	2.7	17.3
Rhodotorula glutinis	b	168	41.7	10.1	1.1	0.2	9.9	1.9	12.6
NRRL YB-252	a, c, d	264	59.0	15.1	1.0	2.0	6.6	5.0	33.1
Cryptococcus curvatus	c, d	192	50.9	16.6	2.9	4.5	17.5	5.7	34.3
NRRL Y-1511	a, b	220	63.0	19.0	4.4	2.6	23.2	5.4	28.4
Lipomyces starkeyi	c	144	46.4	15.6	4.0	1.0	25.6	5.8	37.1
DSM 70296	a, b, d	170	56.1	17.5	5.9	Tr. *	33.7	6.3	36.0
Cryptococcus curvatus	c	144	40.4	13.5	5.6	4.5	41.5	2.8	20.7
ATCC 20509	a, b, d	216	64.0	17.4	8.1	3.2	46.6	3.2	18.4

* Tr. < 0.1 g/L.

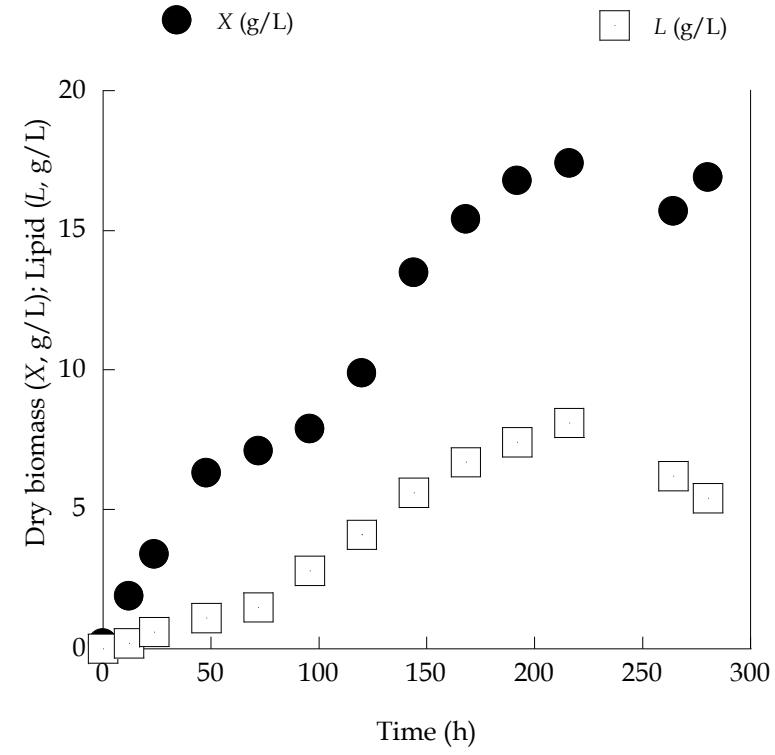
With the exception of the strain *R. glutinis* NRRL YB-252 that gradually and throughout the culture seemed to accumulate total intracellular polysaccharides (IPS) (in accordance with the results achieved during growth on glycerol under nitrogen-limited conditions; see Filippousi et al. [40]), all other yeast strains used seemed to present higher IPS in TDCW values ($Y_{IPS/X}$, % *w*/*w*) at the relative earlier growth steps, and these values seemed to decrease as growth proceeded. Moreover, in several of the tested microorganisms (i.e., strains *C. curvatus* NRRL Y-1511, *R. toruloides* NRRL Y-27012, *R. toruloides* DSM 4444, *L. starkeyi* DSM 70296), appreciable $Y_{IPS/X}$ quantities (i.e., ≥ 35% *w*/*w*) have been reported even at the very early growth steps (i.e., in fermentation time, $t \leq 60$ h) where assimilable nitrogen was found into the medium—or it had barely been exhausted, in accordance with results in which other low-molecular weight hydrophilic carbon sources had been used as

substrates under nitrogen-limited conditions (i.e., crude sucrose, lactose, biodiesel-derived glycerol, etc.; see: [33,40,47]). In contrast, in other yeast strains reported in the literature (yeast species/genera like *Apiotrichum curvatum*, *Yarrowia lipolytica*, other *R. toruloides* strains, *Metschnikowia* sp.) that had been cultivated on hydrophilic carbon sources under nitrogen-limited conditions, it has been seen that accumulation of intracellular polysaccharides inside the cells occurred continuously and without interruption after nitrogen deprivation from the medium, that in some cases occurred simultaneously with lipid accumulation [33,40,51,52].

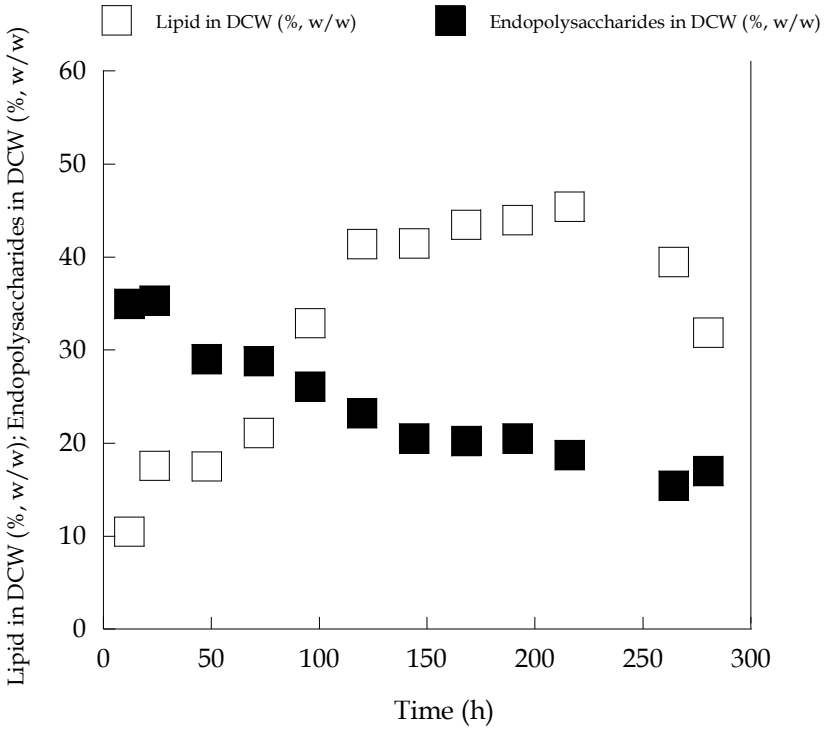
Most of the yeast strains cultivated on xylose produced small quantities of xylitol (Xyl), which in some of the performed trials was reconsumed in order for TDCW to be synthesized. The concentration of the secreted xylitol was not significant (Xyl ≤ 4.5 g/L), and in any case it was drastically lower than the one reported for other eukaryotic microorganisms cultivated on media based on xylose like *Thamnidium elegans* [53], *Ashbya gossypii* [54] and *Mortierella isabellina* [30]. Given that Xyl concentration was not high under the present culture conditions, the biosynthesis of this metabolite was not further studied in the current investigation.

The most promising among the microorganisms tested in the "screening" part of the work was the yeast strain *C. curvatus* ATCC 20509, and the kinetic profile of growth of this strain on commercial-type xylose in shake-flask cultures is depicted in Figure 1a–c. The microorganism presented efficient microbial growth with almost not any lag phase at all occurring during growth. The μ_{max} value, calculated by the formula $\mu_{max} = \frac{\ln(X_1/X_0)}{t_1-t_0}$ (where X_1 was the first experimental point after inoculation and t_1 was the respective time; X_0 was the initial TDCW quantity and $t_0 = 0$ h) was found to be = 0.19 h^{-1}. Moreover, cellular lipids in appreciable quantities were accumulated only after virtual depletion of nitrogen from the growth medium, which occurred at $t \approx 60$ h after inoculation (see and compare evolutions of FAN in Figure 1c and $Y_{L/X}$ in Figure 1b). Interestingly, xylose consumption rate seemed to be uninterrupted despite nitrogen limitation that rapidly occurred in the medium (Figure 1c), in disagreement with results reported for several types of oleaginous microorganisms cultivated on sugars or similarly metabolized compounds under nitrogen-limited conditions, where sugar uptake rate was (much) higher in the balanced growth phase in which nitrogen in significant quantities was found into the medium, compared with that recorded in the lipid-accumulating phase in which nitrogen had been exhausted [30,55–59]. On the other hand, IPS in non-negligible concentrations (i.e., $Y_{IPS/X}$ > 35% *w*/*w*) were recorded at the early growth steps during the presence of assimilable nitrogen into the medium. $Y_{IPS/X}$ values significantly decreased as the fermentation proceeded (Figure 1b,c). This sequential biosynthesis of intracellular polysaccharides and storage lipids that finally becomes an "interplay" between their productions has been originally observed in another *C. curvatus* strain (namely NRRL Y-1511) during its flask cultures on commercial-type lactose. This physiological feature seems to be quite characteristic for the species *C. curvatus*, and it has also been observed, nevertheless less clearly than in the case of *C. curvatus*, for other oleaginous species like *M. isabellina* growing on sugars [30] or *R. toruloides* growing on glycerol [33].

Finally, at the late growth steps and when the concentration of xylose seemed not capable to saturate the microbial metabolic activities, cellular lipids were mobilized in favor of energy creation, in accordance with several reports indicated in the literature for *Mucor circinelloides* [60], *Y. lipolytica* [28,32,41], *M. isabellina* [30,32,44], *Cunninghamella echinulata* [57], *Trichoderma viride* [61], *Candida curvata* [62,63] and other oleaginous strains, suggesting that lipid mobilization (turnover) in the oleaginous microorganisms is a quite common feature observed regardless of the carbon source that was used in order for cellular lipids to be created [22].

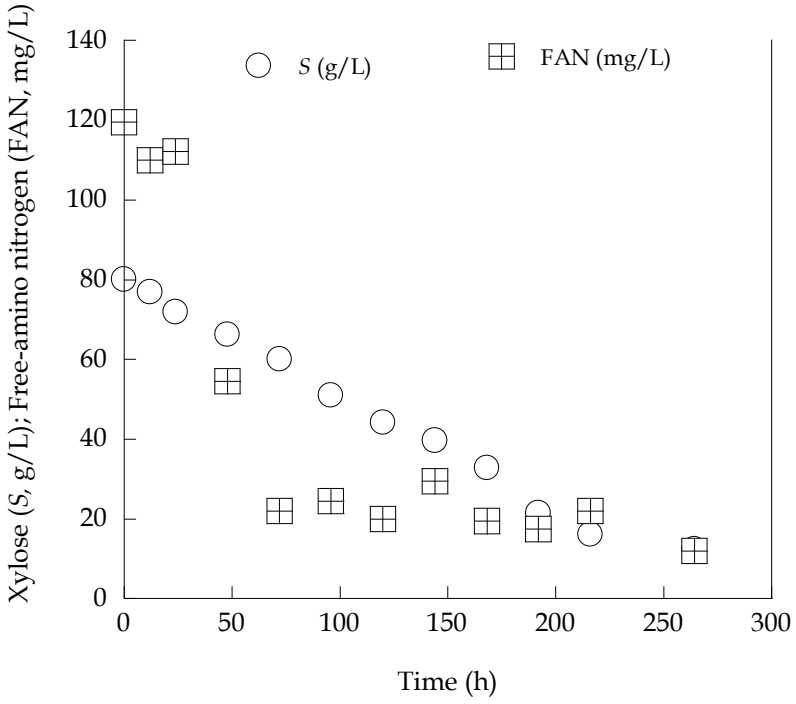


(**a**)



(**b**)

Figure 1. *Cont.*



(**c**)

Figure 1. (**a**) Kinetics of total dry cell weight (TDCW; *X*, g/L) and lipid (*L*, g/L) production, (**b**) lipid in TDCW (%, *w*/*w*) and endopolysaccharides in TDCW (%, *w*/*w*) evolution and (**c**) xylose (*S*, g/L) and free-amino nitrogen (FAN, mg/L) assimilation by *Cryptococcus curvatus* ATCC 20509, during growth on commercial-type xylose in shake-flask experiments under nitrogen-limited conditions. Culture conditions: growth on 250-mL conical flasks filled with the ⅕ of their volume at 180 ± 5 rpm, initial pH = 6.0 ± 0.1, pH ranging between 5.2 and 5.8, incubation temperature 28 °C, initial sugar concentration ≈70 ± 10 g/L. Each experimental point is the mean value of two independent measurements (SE < 15%).

3.2. Lipid Production in High-Xylose Concentration Media Partially Diluted with Olive Mill Wastewaters

In the second part of the current investigation, the most promising of the previously screened strains as regards their lipid-producing capabilities (namely *C. curvatus* ATCC 20509 and *L. starkeyi* DSM 70296), were cultured on media composed of higher S_0 concentrations (adjusted to ≈ 100 g/L), in which the concentration of extracellular nitrogen remained constant (as in the "screening" experiment). The increase of the S_0 concentration in media in which nitrogen availability remained stable, would be attainable to further "boost" the production of SCOs, since higher initial C/N molar ratio media would have been prepared and typically and according to the theory lipid accumulation would have further been stimulated [5,7,8,23]. In these media, OMWs were added in various quantities, in order to partially replace tap water in the fermentations carried out. As previously indicated, this concept was realized within the frame of zero-waste release and water-saving policies that are currently adopted, in a dual scope: To partially replace water by this highly polluted and toxic wastewater, and to potentially perform partial detoxification (i.e., removal of phenolic compounds and color) during lipid production bioprocesses performed. Moreover, it must be stressed that the phenolic compounds that are presented into the OMWs, present significant chemical similarities with recalcitrant phenol-type compounds that are found in various abundant xylose-rich lignocellulose-type wastewaters like spent-sulfite liquor [64–66] and waste xylose mother liquid [1,6]. The composition itself of the media that were currently prepared in order to carry out the lipid production bioprocesses (viz. media containing initial xylose at *c.* 100 g/L, insignificant glucose and fructose quantities (<5 g/L) and variable PC_0 concentrations ranging between 0.0 g/L (no OMWs added) and *c.* 2.0 g/L (60% OMWs and 40% water))

present noticeable similarities with the above-mentioned liquid waste-streams. Therefore, in this part of the work it can be considered among other issues, that lipid production was studied in media mimicking the composition of various types of abundant xylose-containing lignocellulosic-type wastewaters.

The obtained results for *C. curvatus* ATCC 20509 and *L. starkeyi* DSM 70296 cultures performed on xylose-based media blended with OMWs are depicted in Table 2. Concerning the cases of *C. curvatus*, the obtained results indicated that within the range of PCC_0 added into the medium (up to *c.* 2.0 g/L) significant TDCW occurred regardless of the addition of OMWs (and, hence, phenolic compounds) into these media. Moreover, interestingly, it appears that the more the OMWs were added into the medium, the more the $TDCW_{max}$ concentrations increased. In a similar trend, as far as the case of *L. starkeyi* is concerned, addition of OMWs in significant concentrations (viz. PCC_0 up to *c.* 2.0 g/L) did not seem to have serious impact upon the TDCW synthesis by this microorganism, that always remained in high levels ($TDCW_{max} \approx$ 25–26 g/L irrespective of the addition of OMWs), demonstrating its suitability related with biomass production on these types of media, in accordance with results reported for other strains of this species [67,68].

Table 2. Quantitative data of *Cryptococcus curvatus* ATCC 20509 and *Lipomyces starkeyi* DSM 702096 originated from kinetics performed on commercial-type xylose, in nitrogen-limited shake-flask cultures, in which OMWs were added in various concentrations into the medium and the initial sugar (mostly xylose) concentration was adjusted to *c.* 100 g/L. The initial phenolic compounds concentration (PCC_0) is illustrated for all runs carried out. Two different points in the fermentations are represented: (a) when the maximum quantity of total dry cell weight (TDCW—*X*, g/L) was observed; (b) when the maximum quantity of total cellular lipids (*L*, g/L) was observed. Fermentation time (h), quantities of TDCW (*X*, g/L), total lipid (*L*, g/L), total sugar (mostly xylose) consumed (S_{cons}, g/L) and lipid in TDCW ($Y_{L/X}$,% *w*/*w*) are depicted for all the above-mentioned fermentation points. Culture conditions: growth on 250-mL conical flasks filled with the ⅕ of their volume at 180 ± 5 rpm, initial pH = 6.0 ± 0.1, pH ranging between 5.2 and 5.8, incubation temperature 28 °C. Each experimental point is the mean value of two independent measurements (SE < 15%).

PCC_0 (g/L)		Time (h)	S_{cons} (g/L)	*X* (g/L)	*L* (g/L)	$Y_{L/X}$ (%, *w*/*w*)
Cryptococcus curvatus ATCC 20509						
0.00 *	b	168	87.3	21.0	10.0	47.6
	a	215	89.9	23.0	6.0	26.1
1.16	b	144	77.6	20.9	8.4	40.2
	a	215	89.7	24.4	3.6	14.8
1.65	b	240	94.8	24.7	5.0	20.2
	a	336	97.0	25.7	1.8	7.0
1.91	b	192	92.1	23.8	2.5	10.5
	a	216	94.0	27.0	1.6	5.9
Lipomyces starkeyi DSM 702096						
0.00 *	b	216	84.1	25.3	6.0	23.7
	a	239	92.3	26.2	4.4	16.7
1.96	b	239	92.8	21.1	5.9	27.9
	a	255	96.0	25.0	4.0	16.0

*: No olive mill wastewaters (OMWs) added.

It is not a simple task to explain this type of feature of the microbial "resistance"—or, even more, the "boost" of TDCW production in the presence of OMWs in the fermentations carried out. It is widely known that these wastewaters contain phenolic compounds that are recalcitrant and highly toxic substances, the presence of which constitutes the main problem of the safe remediation and disposal of OMWs and similar types of wastewaters [9,20,37,38,69]. On the other hand, it must be pointed out that within the range of PCC_0 found in the current investigation (viz. 0.0 up to 2.0 or even 3.0 g/L),

similar results concerning the "resistance" of TDCW production—or even the "boost" of growth in the presence of OMWs for other yeast strains—has been reported [11,12,16–18,70]. Specifically, the increment of TDCW production by the strain *Saccharomyces cerevisiae* MAK-1 in which OMWs were added in various initial concentrations (additions up to $PCC_0 \approx 3.0$ g/L) was impressive as compared with the blank experiment ($PCC_0 = 0.0$ g/L—no OMWs added) [18]. Likewise, various strains of the nonconventional yeast *Y. lipolytica* noticeably resisted despite the presence of phenolic compounds in significant concentrations (i.e., PCC_0 up to 5.5 g/L) in the media [11,12,17,70,71]. In accordance with the achieved in the present study results, other yeasts belonging to the species *Candida cylindracea*, *C. tropicalis*, *C. albidus* and *Trichosporon cutaneum* seemed to demonstrate remarkable resistance upon the phenolic compounds of OMWs [15,68,72–74]. Likewise, some addition of OMWs into glucose-based media (i.e., OMWs added to 20% or 30% *v*/*v*) seemed to enhance TDCW production not only in yeast cultures but also in other cultures of microbial species like the edible fungi *Lentinula edodes* and *Pleurotus ostreatus* growing on OMW-based media [69,75].

The addition of phenolic compounds into the medium significantly altered the cellular metabolism of *C. curvatus* shifting the intracellular carbon flow towards the synthesis of lipid-free biomass, and significantly decreasing the accumulation of lipid inside the yeast cells (Table 2). When PCC_0 was = 0.0 g/L (no OMWs added), *C. curvatus* produced noticeable SCO quantities (L_{max} = 10.0 g/L corresponding to $Y_{L/X} \approx 48\%$ *w*/*w*) that were reduced when increasing PCC_0 and thus, increasing OMWs amounts were added into the medium. When PCC_0 was adjusted to ≈ 1.2 g/L (viz. trials in which *c.* 100 g/L of xylose were diluted to *c.* 35% *v*/*v* of OMWs and *c.* 65% *v*/*v* of tap water), again the production of SCO was quite satisfactory (L_{max} = 8.4 g/L corresponding to $Y_{L/X}$ = 40.2% *w*/*w*). Further increase of OMWs addition into the medium (i.e., PCC_0 up to ≈2.0 g/L meaning that xylose was diluted to *c.* 60% *v*/*v* of OMW and *c.* 40% of tap water) noticeably decreased both the L_{max} and $Y_{L/X}$ values for *C. curvatus*. On the other hand, SCO production bioprocess for *L. starkeyi* growing on phenol-containing wastewaters enriched with commercial-type xylose seemed to be more "robust" compared with the one of *C. curvatus*; as it was demonstrated, at least for the range of PCC_0 tested in the current submission (PCC_0 up to *c.* 2.0 g/L), lipid production seemed unaffected by the addition of toxic phenolic compounds into the medium (see Table 2). The above-mentioned physiological feature of *L. starkeyi* (meaning, in fact, that lipid production seemed unaffected by the addition of OMWs into the medium, at least until $PCC_0 \approx 2.0$ g/L) together with the fact that $TDCW_{max}$ production was equally not negatively influenced by the addition of the same range of OMWs into the medium, were the main reasons for which the growth of *L. starkeyi* was not tested in intermediate PCC_0 values (i.e., 1.2 or 1.7 g/L), as it happened with *C. curvatus*. Moreover, another interesting result achieved in this second set of experiments, was related with the fact that the highest concentrations of TDCW and total lipids did not occur at the same fermentation time (see Table 2); apparently, the carbon flow that occurred during the fermentation steps after nitrogen limitation led to significant lipid accumulation (that was higher compared with the results reported in Table 1, as it has been anticipated), but finally, cellular lipids were subjected to degradation despite the fact that xylose remained in some non-negligible concentrations into the medium. At the latter growth steps, the low xylose concentration seemed incapable to saturate the metabolic requirements of *C. curvatus* and *L. starkeyi*, leading to consumption of the cellular lipids in favor of lipid-free material, and for this reason TDCW concentration peaked at the end of the cultures (see Table 2).

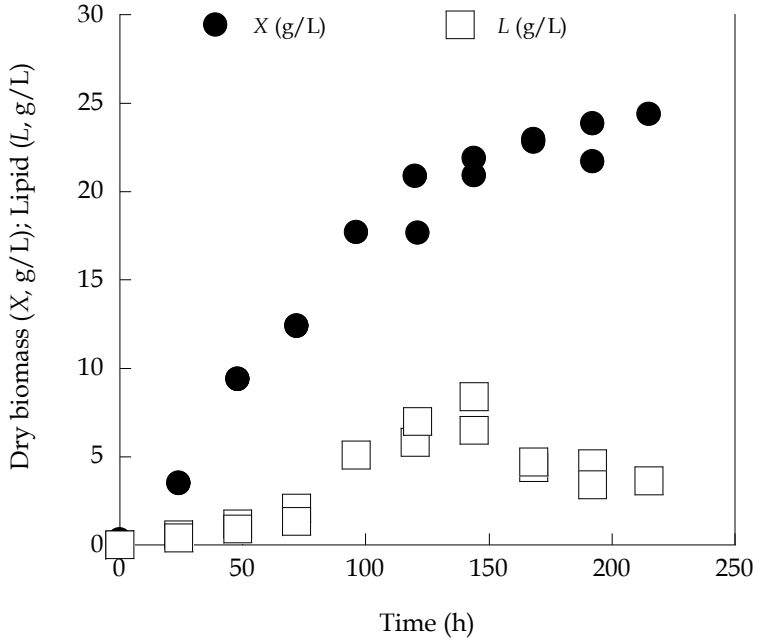
The addition of OMWs into the medium seemed to have contradictory results in relation to several types of oleaginous microorganisms; for instance, addition of PC increased, in some cases noticeably, the quantity of lipids in TDCW for some strains of the yeast *Y. lipolytica* [11,17]. Equally, addition of OMWs in glucose-based cultures of oleaginous Zygomycetes, seemed to enhance the $Y_{L/Xmax}$ values of several oleaginous Zygomycetes, and due to this feature, OMWs had been characterized as a "lipogenic" medium [26]. In the above-mentioned studies, as in the current investigation, OMWs deriving from 3.0-phase extraction systems and containing comparable (and, in general, relatively low) concentrations of sugars were employed as microbial substrates and fermentation waters. Moreover,

these OMWs contained indeed negligible concentrations of residual olive oil, as is the case of the current investigation. This fact excludes any ex novo lipid accumulation process that could be carried out from olive oil found into the growth medium; in the above-mentioned studies as in the current investigation, physiological differences concerning the microbial behavior seemed to be attributed mostly to the PC presence into the medium. Contradictory to these results, the addition of OMWs negatively affected the production of SCO in the strain *L. starkeyi* NRRL Y-11557, than the control experiment (trial on glucose in which no OMWs were added) [68]. Generally, the addition of OMWs can significantly change the spectrum of the final products synthesized by various types of yeasts, compared with the control trials (no OMWs added) [11,12,15,17,70,73]. The kinetics of TDCW and lipid production and total extracellular sugars (mostly xylose) and FAN evolution during growth of *C. curvatus* in media in which PCC_0 was adjusted to ≈ 1.2 g/L is depicted in Figure 2a,b.

The μ_{max} value, calculated as previously was found to be slightly lower than in the previous trial (=0.13 h^{-1}) and this was due to the potential inhibiting effect of the phenol-type wastewater found into the medium. As in the previous set of experiments (see Figure 1a,b) cellular lipids were subjected to biodegradation when somehow low quantities of xylose, incapable to saturate the microbial metabolic requirements, were found into the medium.

Given that *C. curvatus* presented an efficient growth and a quite satisfactory lipid-accumulation in shake-flask cultures in which high initial xylose concentrations were added, whereas the addition of OMWs into the medium negatively affected the process of lipid accumulation, in the next part of the present investigation it was decided to scale-up the culture in a bench top laboratory-scale bioreactor (active volume = 2 L). Commercial xylose (S_0 ≈ 100 g/L) and nitrogen compounds (yeast extract at 3.0 g/L and peptone at 1.0 g/L) were added as in the previous part of the investigation, whereas no OMWs were added into the medium, in order to enhance the maximum the quantity of cellular lipids produced by *C. curvatus*. The obtained results are shown in Figure 3a,b. Compared with the equivalent trial performed in shake-flask mode (see Table 2, entry in which PCC_0 = 0.0 g/L) it may be assumed that xylose uptake rate seemed to be slightly lower in the bioreactor experiment than in the shake-flask trial. This finding is in agreement with results in which filamentous fungi (like *T. elegans* and *M. isabellina*) have been reported to consume more rapidly sugars (including xylose) in shake-flask experiments than in batch bioreactor experiments [44,53,59]. On the other hand—and in accordance with the literature [5,22,23]—the batch bioreactor experiment of *C. curvatus* cultivated on commercial-type xylose revealed $TDCW_{max}$ and L_{max} values that were significantly higher than in the respective shake-flask trial (after 340 h of incubation X_{max} and SCO_{max} quantities achieved were 37.0 and 16.4 g/L with respective $Y_{L/X}$ value = 44.3% *w*/*w*). Moreover, the global yield of SCO produced per quantity of xylose consumed ($Y_{L/S}$) was ≈0.17 g/g (Figure 3b), that is higher than the respective shake-flask experiment (≈0.11 g/g– see results in Table 2, entry in which PCC_0 = 0.0 g/L).

In any case, the production of TDCW and lipids for *C. curvatus* and *L. starkeyi* cultivated on commercial-type xylose and blends of commercial-type xylose and OMWs seemed promising. Various bioreactor experiment studies using batch, fed-batch and continuous strategies have appeared in a number of times in the literature [50,76–81], including in various cases the strains *C. curvatus* ATCC 20509 and *L. starkeyi* DSM 70,296. Comparisons of TDCW and lipid production with data collected from the relevant literature concerning *C. curvatus* and *L. starkeyi* strains are depicted in Table 3. From this table it can be deduced that strains of the species *L. starkeyi* (and also the currently used DSM 70296 strain) can present excellent SCO production in bioreactor experiments [50,76], therefore potentially high lipid production can be achieved by this microorganism cultivated on xylose-based media in bioreactor trials. On the other hand, the reported in the current investigation results by *C. curvatus* ATCC 20509, especially the ones achieved in batch bioreactor trials during growth on commercial-type xylose, were revealed as quite competitive and promising, demonstrating the potential of the microorganism in this type of bioprocesses.



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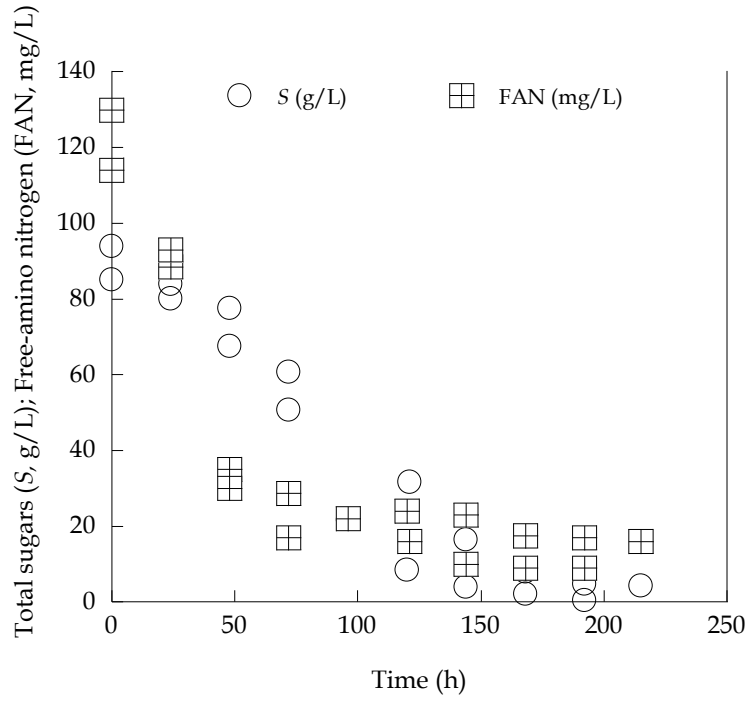
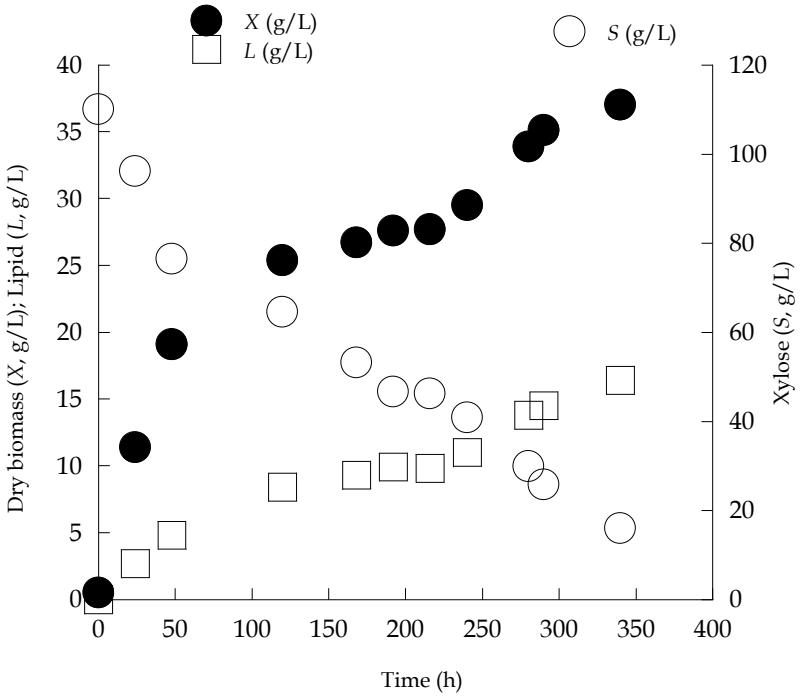


(**b**)

Figure 2. (**a**) Kinetics of total dry cell weight (TDCW; *X*, g/L) and lipid (*L*, g/L) production and (**b**) xylose (*S*, g/L) and free-amino nitrogen (FAN, mg/L) assimilation by *Cryptococcus curvatus* ATCC 20509, during growth on commercial-type xylose in media diluted with olive mill wastewaters, at initial phenol-content concentration ≈1.2 g/L in shake-flask experiments under nitrogen-limited conditions. Culture conditions: growth on 250-mL conical flasks filled with the ⅕ of their volume at 180 ± 5 rpm, initial pH = 6.0 ± 0.1, pH ranging between 5.2 and 5.8, incubation temperature 28 °C, initial sugar concentration ≈100 ± 10 g/L. Each experimental point is the mean value of two independent measurements (SE < 15%).



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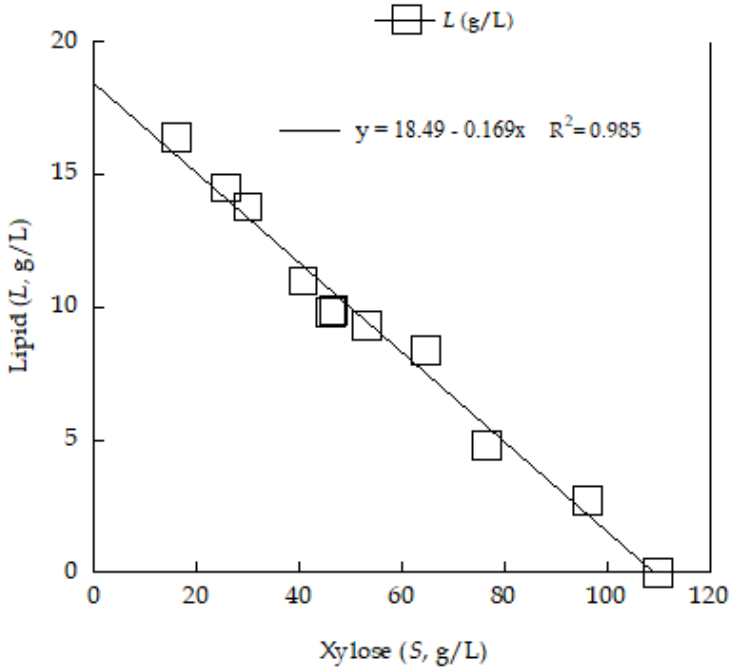


(**b**)

Figure 3. (**a**) Kinetics of evolution of total dry cell weight (TDCW; *X*, g/L), lipid (*L*, g/L) and xylose (*S*, g/L) and (**b**) lipid produced (*L*, g/L) vs. remaining xylose (*S*, g/L) by *Cryptococcus curvatus* ATCC 20509, during growth on commercial-type xylose in batch bioreactor experiment under nitrogen-limited conditions. Culture conditions: growth on laboratory-scale bioreactor (active volume 2.0 L), agitation rate 450 ± 5 rpm, incubation temperature 28 °C, aeration rate up to 1.5 vvm, initial xylose concentration (S_0) ≈100 g/L, pH ranging between 5.9 and 6.1, oxygen saturation ≥ 20% (*v*/*v*) for all growth phases. Each experimental point is the mean value of two independent measurements (SE < 15%).

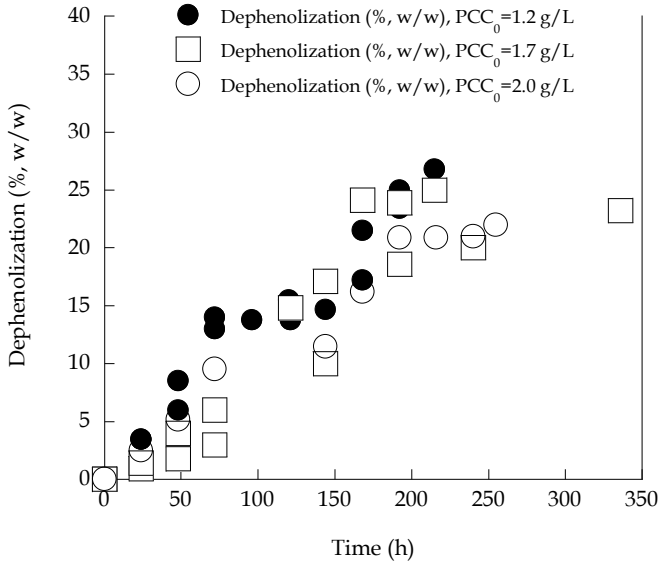
Table 3. Total dry cell weight (TDCW, g/L) and lipid in TDCW ($Y_{L/X}$,% *w*/*w*) values obtained by (a) *Cryptococcus curvatus* and (b) *Lipomyces starkeyi* strains growing on several carbon sources and fermentation configurations.

Strain	Culture Mode	Carbon Source	TDCW (g/L)	$Y_{L/X}$ (%, *w*/*w*)	Reference
(a)					
Cryptococcus curvatus					
D	Continuous bioreactor	Glucose Xylose	13.5 15.0	29.0 37.0	Evans and Ratledge [77]
ATCC 20509	Batch bioreactor Continuous recycling	Whey permeate	21.6 85.0	36.0 35.0	Ykema et al. [78]
	Batch bioreactor	Prickly pear juice	10.9	45.8	Hassan et al. [79]
	Fed-batch bioreactor	Glycerol (pure)	118.0	25.0	Meesters et al. [80]
	Fed-batch bioreactor	Glycerol (crude)	32.9	52.9	Liang et al. [81]
	Fed-batch bioreactor	Glycerol (crude)	44.9	49.0	Cui et al. [82]
	Single-stage continuous	Acetic acid	5.1	66.4	Gong et al. [83]
NRRL Y-1511	Shake flasks	Lactose (commercial)	14.5	29.7	Tchakouteu et al. [47]
	Shake flasks Shake flasks	Glucose Xylose (pure)	13.2 13.3	19.7 38.3	Harde et al. [48]
ATCC 20509	Continuous bioreactor	Acetic acid	26.7	48–53	Béligon et al. [84]
	Shake flasks	WPHL	17.3	52.5	Zhou et al. [85]
	Fed-batch bioreactor	Cheese whey	66.8	49.6	Kopsahelis et al. [86]
ATCC 20509	Shake flasks	Xylose (commercial) Xylose/OMWs	21.0 20.9	47.6 40.2	Present study
	Batch bioreactor	Xylose (commercial)	37.0	44.3	
(b)					
Lipomyces starkeyi					
DSM 70295	Shake flasks	Glucose Glucose/sewage sludge	9.4 9.3	68.0 72.3	Angerbauer et al. [87]
AS 2.1560	Batch bioreactor	Glucose	30.0	46.0	Liu et al. [88]
CBS 187	Shake flask	Xylose (pure)	12.5	80.0	Oguri et al. [89]
DSM 70296	Fed-batch bioreactor Batch bioreactor	Glucose/xylose SCBHL	82.4 13.9	46.9 26.7	Anschau et al. [76]
AS 2.1560	Fed-batch bioreactor	Xylose (pure)	94.7	65.5	Lin et al. [90]
CBS 1807	Shake flasks	Glucose/fructose SSJc	12.3 21.7	47.3 29.5	Matsakas et al. [91]
DSM 70296	Fed-batch bioreactor Shake flasks	FRWHL/glucose FRWHL	109.8 30.5	57.8 40.4	Tsakona et al. [50]
	Shake flasks	Glycerol (crude)	34.4	35.9	Tchakouteu et al. [39]
NRRL Y-11557	Shake flasks	Glucose/OMWs	9.5	24.5	Dourou et al. [68]
DSM 70296	Shake flasks	Xylose (commercial) Xylose/OMWs	25.3 21.1	23.7 27.9	Present study

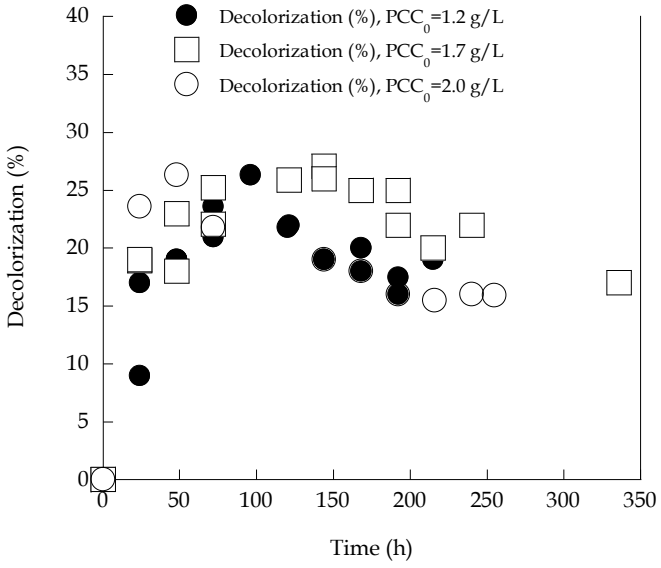
WPHL—waste paper hydrolysate; SCBH—sugarcane bagasse hydrolysate; SSJc—sweet sorghum juice; FRWH—flour-rich waste hydrolysate; OMWs—olive mill wastewaters.

Concerning the trials in OMW-based media, irrespective of the PCC_0 imposed into the medium, *C. curvatus* proceeded to a gradual dephenolization of the medium, reaching to a total phenolic content removal of ≈25–28% *w*/*w* (Figure 4a). On the other hand, decolorization process was not "parallel" with that of dephenolization (Figure 4b), reaching much more rapidly to a color removal "plateau" (*c.* 25% of decolorization), whereas, interestingly, at the late fermentation steps (i.e., t > 200 h), although dephenolization constantly occurred, the color into the medium seemed to be more "dense" than in the earlier growth steps. As far as the detoxification process led by *L. starkeyi* was concerned, the kinetic profile of phenol removal was almost identical with the ones recorded for *C. curvatus*

(see Figure 3a). In contrast—and despite the non-negligible dephenolization that occurred—*L. starkeyi* did not remove at all color from the wastewater. It may be assumed, therefore, that color and phenol removal from phenol-containing wastewaters, seem processes that are not obligatorily implicated to each other, in agreement with results recorded for the detoxification of wastewaters performed by edible and medicinal mushrooms [37,69], several types of yeasts like strains of *Y. lipolytica* and *S. cerevisiae* [11,16,18,70] or even crude enzymes deriving from mushroom cultivations [92].



(**a**)



(**b**)

Figure 4. Kinetics of (**a**) dephenolization (%, *w/w*) and (**b**) decolorization (%) performed by *Cryptococcus curvatus* ATCC 20509, during growth on commercial-type xylose in media diluted with olive mill wastewaters, at initial phenol-content concentrations ≈1.2 g/L, ≈ 1.7 g/L and ≈ 2.0 g/L, in shake-flask experiments under nitrogen-limited conditions. Culture conditions: growth on 250-mL conical flasks at 180 ± 5 rpm, initial pH = 6.0 ± 0.1, pH ranging between 5.2 and 5.8, incubation temperature T = 28 °C, initial sugar concentration ≈100 ± 10 g/L. Each experimental point is the mean value of two measurements (SE < 15%).

Yeast strains compared to higher fungi, do not possess the mechanisms of producing the appropriate extracellular oxidases [10,13,92] to break down phenolic compounds that are found in several phenol-containing wastewaters [37,38,69,92]. Oxidative enzymes produced by higher fungi in these processes include but are not limited to lignin peroxidase (*LiP*, E.C. 1.11.1.14), manganese-dependent peroxidase (*MnP*, E.C. 1.11.1.13), phenol oxidase (laccase) (*Lac*, E.C. 1.10.3.2) [37,38,69,92]. On the other hand, besides OMWs, phenol-containing wastes are nowadays produced in significant quantities from several industrial processes (e.g., coal conversion, petroleum refining, sugar refining, paper production processes, etc.) [7,10,37], therefore these "detoxification" and "remediation" bioprocesses present a constantly increasing scientific and industrial importance. The last years, in an increasing number of reports, a non-negligible reduction of phenolic-type compounds has been observed in cultures performed by yeast species like *C. cylindracea*, *C. tropicalis*, *L. starkeyi* and *T. cutaneum* [15,67,68,73,74,93]. Since, as previously indicated, yeasts lack the existence of phenol-oxidizing enzymes [10,13], the OMW decolorization and removal of phenol compounds by yeasts should not be achieved by such mechanisms. Potentially, adsorption of phenolic compounds in the yeast cells may exist [11]. It could be also supposed that partial utilization of phenolic compounds as carbon source by the microorganism may occur [67,73,74].

3.3. Cellular Lipid Analysis

All screened strains were analyzed concerning the fatty acid (FA) composition of their total lipids at the stationary growth phase (FA composition analysis performed at t = 120–150 h after inoculation) (Table 4). In agreement with the literature (for critical reviews see: [3,5,22,23,25]) the principal FAs found in variable quantities were mainly oleic acid (Δ9C18:1) and palmitic acid (C16:0). Other FAs like stearic (C18:0) palmitoleic (Δ9C16:1) and linoleic (Δ9,12C18:2) were found in lower concentrations, whereas poly-unsaturated FAs containing >2 double bonds (i.e., α- and γ-linolenic acid, arachidonic acid, etc.) were not detected, since these compounds are the principal storage lipophilic compounds in oleaginous fungi and algae [25–27]. The FA compositions of lipids produced by other *C. curvatus*—or generally, other nonconventional yeast species/genera employed as cell factories amenable to produce SCOs (like *Y. lipolytica*, *Rhodosporidium* sp., *Lipomyces* sp., etc.) during growth on sugars or other hydrophilic substrates (like glycerol)—can present differences that reflect on the microbial growth stage, the incubation temperature, the initial molar ratio C/N and the initial concentration of substrate used without any systematic common effect on the modification of cellular FAs by the above-mentioned parameters [33,39,40,47,48,58,67,68,81–87]. On the other hand, SCOs containing increased concentrations of the FA Δ9C18:1 and non-negligible ones of the FAs C16:0 and Δ9,12C18:2 (as is the case of the yeast lipids produced in the current investigation; see Table 4) constitute perfect fatty materials amenable to produce high-quality "2nd generation" biodiesel [3–5,7,8,22,25].

In the present investigation, the addition of OMWs into the culture medium, did not seem to bring significant modifications in the FA composition of the total cellular lipids produced by *C. curvatus* (Table 5a) and *L. starkeyi* (Table 5b). As previously, the main cellular FAs were the Δ9C18:1 and C16:0, therefore, SCOs produced by *C. curvatus* and *L. starkeyi* cultivated on commercial xylose/OMW blends were suitable materials to be converted into nonconventional biodiesel. The FA C16:0 seemed to be presented in slightly higher concentrations in the cellular lipids of *L. starkeyi* compared with these of *C. curvatus*. In disagreement with the results indicated in the current investigation, addition of OMWs, as indicated by the PCC_0 that was comparable with the current investigation, seemed to significantly increase the quantity of the cellular FA Δ9C18:1 decreasing that of C18:0 and Δ9C16:1 for various strains of the yeasts *S. cerevisiae* and *Y. lipolytica* [11,12,16–18]. It had been postulated that the presence of microbial inhibitors like gallic acid, caffeic acid, polyphenols, etc., could perform potential activation of the cellular Δ9 desaturase (catalyzing the reaction of conversion of the FA C18:0 to the FA Δ9C18:1) [11,18]. However, as shown in the current investigation (Table 5a,b), the mentioned feature of the yeasts *S. cerevisiae* and *Y. lipolytica* in not observed for *L. starkeyi* and *C. curvatus*.

Table 4. Fatty-acid composition of the cellular lipids produced by yeast strains cultivated on commercial-type xylose employed as sole carbon source in shake-flask experiments (S_0 concentration ≈ 70 g/L). Time of fermentation for the determination of the fatty-acid composition was at the stationary growth phase (between 120 and 150 h after inoculation). Culture conditions as in Table 1.

	Fatty-Acid Composition of Yeast Lipids (%, *w/w*)					
Yeast Strain	**C16:0**	**Δ9C16:1**	**C18:0**	**Δ9C18:1**	**Δ9,12C18:2**	**Others**
Rhodosporidium toruloides DSM 4444	29.5	0.6	7.0	51.6	7.7	3.6
Rhodosporidium toruloides NRRL Y-27012	28.4	0.5	6.9	53.7	7.5	3.0
Rhodotorula glutinis NRRL YB-252	20.5	1.5	2.0	55.5	9.0	11.5
Cryptococcus curvatus NRRL Y-1511	22.5	0.5	14.0	49.9	13.0	0.1
Lipomyces starkeyi DSM 702096	28.9	5.0	5.4	50.1	5.5	5.1
Cryptococcus curvatus ATCC 20509	22.5	0.9	9.0	51.1	10.8	5.7

Table 5. Fatty-acid composition of cellular lipids of *Cryptococcus curvatus* ATCC 20509 (a) and *Lipomyces starkeyi* DSM 702096 (b) during growth on commercial-type xylose, in nitrogen-limited shake-flask cultures, in which OMWs were added in various concentrations into the medium and the initial sugar (mostly xylose) concentration was adjusted to *c.* 100 g/L. The initial phenolic compounds concentration (PCC_0) is also illustrated for all runs carried out. LE is the late exponential phase (t ≈ 48 h) and S is the stationary phase (t ≈ 150–180 h for *C. curvatus* and t = 215 h for *L. starkeyi*). Culture conditions as in Table 2.

(a) *Cryptococcus curvatus*		**Fatty-Acid Composition of Cellular Lipids (%, *w/w*)**				
PCC_0 (g/L)	**Fermentation Period**	**C16:0**	**C18:0**	**Δ9C18:1**	**Δ9,12C18:2**	**Others**
0.0	LE	28.0	8.6	50.0	10.5	2.9
	S	24.0	11.0	54.3	7.0	3.7
1.16	LE	25.3	11.4	46.3	5.4	11.6
	S	21.5	13.0	54.0	7.5	4.0
1.65	LE	25.0	12.0	49.0	6.1	7.9
	S	19.7	13.7	54.4	8.2	4.0
1.91	LE	24.8	12.2	51.2	6.8	5.0
	S	20.1	13.9	54.0	8.9	3.1
(b) *Lipomyces starkeyi*		**Fatty-Acid Composition of Cellular Lipids (%, *w/w*)**				
PCC_0 (g/L)	**Fermentation Period**	**C16:0**	**C18:0**	**Δ9C18:1**	**Δ9,12C18:2**	**Others**
0.0	LE	31.5	3.9	49.0	5.8	9.8
	S	32.0	5.0	53.0	2.7	7.3
1.96	LE	32.3	4.7	49.7	6.6	6.7
	S	33.4	6.3	52.5	2.9	4.9

C. curvatus lipid was mainly composed of neutral lipids (NLs) (85–88% *w/w* of cellular lipids) whereas smaller quantities of glycolipids plus sphingolipids (G + S) (10–13% *w/w* of cellular lipids) principally phospholipids (PLs) (*c.* 2.0% *w/w* of cellular lipids) were quantified, in an analysis performed at the stationary (and, therefore, "oleaginous") phase (t ≈ 170–190 h after inoculation) (Table 6a,b). In most instances, in the case of oleaginous microorganisms, NLs are mainly composed of triacylglycerols (TAGs) and to lesser extent steryl esters, while low concentrations of mono- and diacylglycerols (MAGs and DAGs) are identified [7,8,23]. It is also noted that NLs have been revealed to be the major component of the cellular lipids irrespective of the fact of significant or insignificant accumulation of lipids in many yeast species like *R. toruloides*, *Y. lipolytica*, *Pichia membranifaciens*, etc., when growth is performed on sugars or glycerol under nitrogen-limited conditions [32,41,58,94].

(Finally, *C. curvatus* ATCC 20509 stored total lipids (extracted with the aid of chloroform/methanol 2/1 *v/v* mixture) that were mainly composed of triacylglycerols (TAGs) (Figure 5), in accordance with many results reported for various oleaginous yeasts [4,33,94] and fungi [32,59].

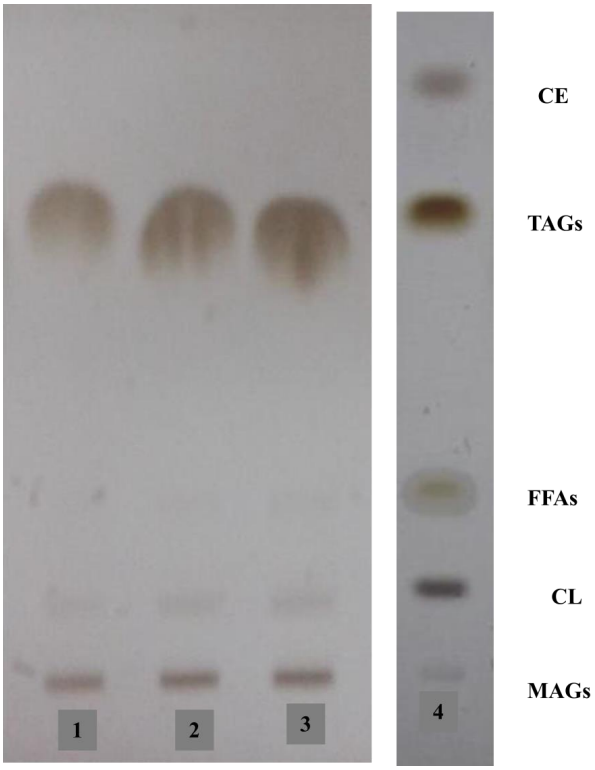


Figure 5. TLC analysis of the "crude" "Folch" extract (lanes 1, 2 and 3) of *Cryptococcus curvatus* ATCC 20509 lipid produced during growth on xylose in shake-flask experiments (S_0 concentration ≈100 g/L), *c.* 170 h after inoculation. Lane 1 corresponds to deposited sample of 4 μL, lane 2 of 6 μL and lane 3 of 8 μL. Total lipids were diluted to chloroform/methanol 2/1 *v/v* solution, at concentration ≈10 mg/mL. Lane 4 corresponds to a mixture of neutral lipid standards (monononadecanoin—MAG, cholesterol—CL, oleic acid—FFA, glyceryl trioleate—TAG and cholesteryl linoleate—CE). Solvents: petroleum ether/diethyl ether/glacial acetic acid (70:30:1, *v/v/v*); time of run 45 min (16 cm above the origin); intermediate drying at room temperature for 15–30 min. Detection system: H_2SO_4:H_2O 1:1 (*v/v*) at 110–120 °C for 30 min. Culture conditions as in Table 2.

Table 6. Quantities (in %, *w/w*) of neutral lipid (NL), sphingolipid and glycolipid (G + S) and phospholipid (PL) fractions during lipid accumulation phase of *Cryptococcus curvatus* ATCC 20509 cultivated on commercial xylose at S_0 ≈ 100 g/L without OMWs added (PCC_0 = 0.0 g/L) in shake-flask experiment, *c.* 170 h after inoculation (a) and batch bioreactor experiment, *c.* 190 h after inoculation (b) and fatty-acid composition (in%, *w/w*) of the mentioned lipid fractions as compared with the fatty-acid composition of total lipid for the relevant fermentation point. Culture conditions for the shake-flask trials as in Table 2, for the batch bioreactor trial as in Figure 3.

(a) Flask Experiment					
	% *w/w*	**C16:0**	**C18:0**	**Δ9C18:1**	**Δ9,12C18:2**
Total lipid		26.0	9.7	53.2	7.2
NLs	85.2	29.0	6.5	50.8	10.8
G+S	12.6	26.0	7.2	53.4	6.1
PLs	2.2	22.1	8.0	59.0	7.0
(b) Bioreactor Experiment					
	% *w/w*	**C16:0**	**C18:0**	**Δ9C18:1**	**Δ9,12C18:2**
Total lipid		32.1	6.0	54.4	2.9
NLs	88.1	40.2	4.8	49.4	3.0
G+S	10.0	37.3	8.8	49.3	2.1
PLs	1.9	26.4	5.1	61.4	4.0

4. Conclusions

Wild-type yeast strains were screened towards their ability to convert commercial xylose, a low-cost carbon source deriving from lignocellulosic biomass, into SCO. Trials were carried out with initial xylose at *c.* 70 g/L using six yeast strains of the species *Rhodosporidium toruloides*, *Lipomyces starkeyi*, *Rhodotorula glutinis* and *Cryptococcus curvatus* and the most promising results as regards lipid production were recorded for the strains *C. curvatus* ATCC 20509 and *L. starkeyi* DSM 70296. In the next step, media presenting high xylose quantities (≈100 g/L) in which OMWs were added in various concentrations, were employed as fermentation media. These media mimic various abundant xylose-rich lignocellulose-type wastewaters like spent-sulfite liquor and waste xylose mother liquid. Both *C. curvatus* and *L. starkeyi* presented interesting TDCW production. In the media without OMWs added, *C. curvatus* produced SCO = 10 g/L (lipid in TDCW ≈ 48% *w*/*w*. Scale-up in laboratory-scale bioreactor clearly increased the production of TDCW and SCO synthesized by *C. curvatus*; the values for both biomass and lipids produced in bioreactor experiments (37.0 g/L and 16.4 g/L respectively) were among the very high ones achieved in the international literature for this type of conversion (commercial xylose towards SCO) demonstrating the high potential of this strain on the mentioned bioprocess.

In media presenting increasing phenolic compounds, lipid in TDCW values decreased whereas shift towards the creation of lipid-free material was observed. In contrast, lipid production bioprocess was significantly unaltered by the presence of phenolic compounds into the medium for *L. starkeyi* (in all cases lipids in TDCW quantities 24–28% *w*/*w*, similar to the blank experiment were recorded). Interesting dephenolization occurred for both yeast strains, irrespective of the initial concentration of the phenolic compounds found into the medium. On the contrary, decolorization of the wastewater occurred only by *C. curvatus*.

Total lipids of all yeasts tested were mainly composed of the FAs oleic and palmitic and to lesser extent linoleic and stearic, found in variable concentrations and constituting perfect fatty materials amenable for the synthesis of high-quality biodiesel. In *C. curvatus*, lipids were mainly composed of nonpolar fractions (i.e., triacylglycerols) whereas polar lipids (i.e., phospholipids, glyco + sphingolipids) were found at drastically lower concentrations.

Concluding, both *C. curvatus* and *L. starkeyi* are robust TDCW- and SCO-producing microorganisms during growth on xylose and xylose/OMWs blends. The current submission has demonstrated the potential of the bioconversion of these compounds or also residues mimicking these substrates (i.e., abundant xylose-containing wastewaters) into yeast biomass and lipid with satisfactory productions and conversion yields.

Author Contributions: Conceptualization, S.P. and A.K.; methodology, S.P.; software, S.P.; validation, S.P., A.K. and E.X.; formal analysis, E.X.; investigation, E.X and I.G.; resources, S.P.; data curation, S.P.; writing—original draft preparation, E.X.; writing—review and editing, S.P and A.C.; visualization, E.X.; supervision, S.P.; project administration, S.P.; funding acquisition, S.P. and A.K. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflicts of interest.

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