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Yeast Lipids from Crude Glycerol Media and Utilization of Lipid Fermentation Wastewater as Maceration Water in Cultures of Edible and Medicinal Mushrooms

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Abstract: Four wild "red" yeast strains (*Rhodosporidium kratochvilovae* FMCC Y70, *R. toruloides* NRRL Y-27013, *R. toruloides* NRRL Y-17902 and *R. toruloides* NRRL Y-6985) were cultured in shake flasks on industrial glycerol at an initial substrate (Gly_0) concentration ≈ 50 g/L under nitrogen limitation. Strains NRRL Y-27013, NRRL Y-17902 and NRRL Y-6985 presented appreciable dry cell weight (DCW) and lipid synthesis (DCW up to 18–19 g/L containing lipids in quantities ≈ 47%, *w*/*w*). Strains NRRL Y-27013 and NRRL Y-6985 were further tested in higher Gly_0 concentrations (≈90 g/L and ≈110 g/L) with the same initial nitrogen quantity as in the first ("screening") experiment. Both strains, despite the high Gly_0 concentrations and C/N ratios (up to 120 moles/moles) imposed, presented significant DCW production (up to c. 29.0–29.5 g/L). Yeast biomass contained significant lipid (42–43%, *w*/*w*) and endopolysaccharide (up to 42%, *w*/*w*) quantities. Both lipids and endopolysaccharide quantities (in % *w*/*w*) noticeably increased as a response to the imposed nitrogen limitation. Lipids containing mainly oleic and palmitic acids constituted ideal candidates for biodiesel synthesis. Thereafter, the wastewaters derived from the lipid production process (lipid fermentation wastewaters—LFWs) were used as maceration waters in cultivations of edible and medicinal fungi, where novel (non-conventional) substrates were used in the performed cultures. CW (coffee residue + wheat straw), CB (coffee residue + beech wood shavings), OW (olive crop + wheat straw), OB (olive crop + beech wood shavings), RW (rice husk + wheat straw) and RB (rice husk + beech wood shavings) were soaked/sprayed with LFWs or tap water and utilized in the cultivation of *Pleurotus*, *Ganoderma* and *Lentinula* mushrooms. The impact of LFWs on the mycelial growth rate (mm/d) and biomass production was evaluated. The results show that regardless of the wetting method, the highest growth rates (6.2–6.6 mm/d) were noticed on RW and RB for *Pleurotus eryngii* and *Ganoderma resinaceum*, on OW, OB and RW for *Ganoderma applanatum* and on RW, OW and OB for *Lentinula edodes*. Nevertheless, high biomass production was obtained on substrates soaked with LFWs for *Pleurotus ostreatus* (RW: 443 mg/g d.w.), *L. edodes* (RB: 238 mg/g d.w.) and *Ganoderma lucidum* (RW: 450 mg/g d.w.). Overall, this study demonstrates the possibility of the industrial conversion of low-value agro-waste to mycelial mass and eventually to important food products.

Keywords: glycerol; intra-cellular lipids; intra-cellular polysaccharides; glucosamine; biomass; oleaginous yeasts; *Rhodosporidium toruloides*; *Pleurotus*; *Ganoderma*; *Lentinula*

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1. Introduction

One of the most important priorities in the utilization of microorganisms as sources of lipids is focused upon their ability to convert several by-products into microbial lipids ("single-cell oils" (SCOs), viz. oils produced by microorganisms) [1–3]. Although the

cost of these lipids was higher than that of traditional animal fats or vegetable oils, SCOs were initially used to replace expensive fatty materials; however, currently, due to the global increase in food cost, SCOs (mainly yeast lipids) can be used as substitutes for common plant oils for the fabrication of biodiesel [1–7]. Furthermore, the huge expansion of biodiesel (viz. FA alkyl-esters) has resulted in a significant rise in the production of glycerol, which is the main side-product of the trans-esterification reaction process. As a result, an important decrease in the price of glycerol occurred last year, and the topic of its valorization has become crucial for biotechnological industries [2,5,7,8]. The utilization of oleaginous yeasts in the conversion of crude glycerol closes the loop of biodiesel production since the principal residue of this industrial activity (viz. crude glycerol) is transformed into triacylglycerols that subsequently will be converted into "second-generation" biodiesel, a process with obvious economic and ecological interest [2,5–8].

In the current study, biodiesel-derived glycerol was utilized as a substrate in "red" non-conventional and non-extensively studied yeast strains (belonging to the genus *Rhodosporidium*), in culture conditions enabling the production of SCOs (viz. fermentations carried out under conditions of limited nitrogen). Under the biorefinery concept, the zero-waste release approach and the water-saving optic recently adopted by various authorities and funding bodies, the wastewaters deriving from the lipid production process (viz. lipid fermentation wastewaters-LFWs) were utilized as maceration waters in innovative solid-state fermentation (SSF) processes performed using edible and pharmaceutical mushrooms. The treatment of LFWs may increase the whole SCO bioprocess cost given that in several cases, these waters can potentially contain non-assimilated and non-used fixed carbon sources (specifically if increased initial sugar quantities are used for the SCO synthesis to be performed). Moreover, LFWs certainly contain (potentially important) quantities of salts [2,4]. These salt-containing wastewaters can be used as maceration waters for the cultivation of edible and pharmaceutical fungi since several of them can present significant growth with or without salts in the culture media [9–13].

Mushrooms may produce several non-specific lignocellulosic enzymes that affect their growth and development [9,14,15]. *Pleurotus*, *Lentinula* and *Ganoderma* species, widely cultivated worldwide, are effective lignocellulosic residue bio-converters, resulting in mushrooms with unique biological and pharmacological properties [9,16–22]. During fermentation and substrate colonization, mushrooms obtain the nutrients required for their growth, mainly carbon and nitrogen sources, minerals and vitamins (these are possibly contained in LFW) [23–25] that along with substrate composition (amount of cellulose, lignin, etc.) may affect both the mycelial growth and mushroom production, and therefore, their evaluation is needed prior to cultivation [26–28].

Additionally, the process of mushroom cultivation has many advantages, as through the recycling or degradation of several agro-industrial wastes (e.g., olive mill waste, coffee residues, rice husk), an alternative way to reduce these impacts is generated and by turning these wastes into valuable products and food, environmental balance and economic prosperity are provided in the frame of a zero-waste economy [26,29–31]. On the other hand, very often, new methods and techniques in mushroom cultivation are proposed, including innovations in substrate preparation and cultivation conditions, to improve their productivity and quality [9,11,32,33]. For example, Dedousi et al. [11], with the addition of oils, nitrogen and calcium salts, achieved a shorter incubation period, higher biological efficiency and high-quality mushrooms. Moreover, the ability of various wastes to serve as substrates is often examined by utilizing large glass tubes and measuring the fungal growth rate (mm/day, Kr), as cultivating mushrooms in "bag-logs" is time-consuming. This technique has been successfully used by many researchers to determine the mycelial growth and fructification in a variety of fungal species, including *Agrocybe aegerita*, *Volvariella volvacea*, *Pleurotus* spp. and *L. edodes* [26,34]. Also, the mycelial mass can be estimated indirectly at the end of the colonization period using the glucosamine content of the fungal cell wall [11,27,35,36]. Chitin is a structural polysaccharide of the fungal cell wall and is composed of β(1,4)-linked units of N-acetyl-D-glucosamine. Because of their

antibacterial and antioxidant qualities, chitin and chitosan are useful compounds with diverse uses, for food and medicinal purposes [37]. Chitin synthesized through fungal SSFs has been proposed, therefore, as an alternate source, as glucosamine may be obtained under controlled conditions utilizing a simple extraction process [38,39]. Apart from that, the determination of both Kr and biomass is necessary for the evaluation of various residues and wastes as potential substrates for high-yield mushroom cultivation.

Therefore, in the present study, four "red" not previously extensively studied yeast strains were used as cell factories amenable to turning biodiesel-derived glycerol into SCOs. The synthesis of other intra-cellular metabolites (i.e., polysaccharides) was also evaluated. Then, LFWs constituted the wetting agents in the SSF of several strains of edible and pharmaceutical fungi with agricultural residues, non-conventional in mushroom cultivation. The purpose of this study was first to estimate the possibility of using the lipid fermentation wastewater from *Rhodosporidium toruloides* instead of tap water during the SSFs of mushrooms. Then, to simplify the procedure and gain time, spraying was used as a wetting technique for residues prior to substrate preparation. For this purpose, three agro-industrial residues not extensively studied (e.g., olive crop residues, rice husk and coffee residue) along with various species of the higher fungi with dietary and/or medicinal properties, such as *Pleurotus*, *Ganoderma* and *Lentinula*, were evaluated for their bioconversion efficacy during small scale SSF (in glass tubes). In this work, the main growth parameters (*viz*. colonization rates, mycelial mass production) were noted, and the effect of substrate components and cultivation handling on them was considered. Finally, further investigation of the impact of LFW on selected substrates and mushroom strains regarding carposome production and metabolic compound synthesis (e.g., polysaccharides, proteins, antioxidants and lipids) is currently taking place.

2. Materials and Methods

2.1. Submerged Cultures of Yeasts for Lipid Production

In the present study, the "red" yeast strains *Rhodosporidium kratochvilovae* FMCC Y-70, *R. toruloides* NRRL Y-27013, *R. toruloides* NRRL Y-17902 and *R. toruloides* NRRL Y-6985 were used. The strain coded FMCC was a new strain deriving from the Laboratory of Food Microbiology and Biotechnology (Agricultural University of Athens, Athens, Greece), isolated from gilt-head sea bream (*Sparus aurata*) and characterized [40]. Strains coded NRRL Y were purchased by the ARS Culture Collection (Peoria, IL, USA). All four mentioned strains have been scarcely employed in works related to their growth opportunity on substrates containing glycerol and their potential for producing microbial lipids. According to Abeln and Chuck [2], the most frequently studied strains of the species *R. toruloides* in relation to their lipid production properties are the strains DSM 4444 (14% of the published papers on the topic of SCO production by *R. toruloides* strains), AS 2.1389 (12% of the published papers) and ATCC 10788 (7% of the published papers), while concerning the strains deriving from the ARS Culture Collection, only the strain NRRL Y-1091 has been studied in some cases in relation to its SCO potential (7% of the published papers) [2]. Therefore, the present study is indeed one of the first in the literature demonstrating the potential of lipid production by the mentioned wild-type yeast strains.

All strains were maintained on yeast peptone dextrose agar (YPDA) supplemented with malt extract, at $T = 4.0 \pm 0.5$ °C, and were sub-cultured every 2 months to maintain their viability. Experiments regarding the production of SCOs were carried out in submerged cultures in media with the following salt composition (in g/L) [41]: KH_2PO_4, 7.0; Na_2HPO4, 2.5; $MgSO_4 \cdot 7H_2O$, 1.5; $CaCl_2 \cdot 2H_2O$ 0.15; $FeCl_3 \cdot 6H_2O$, 0.15; $ZnSO_4 \cdot 7H_2O$, 0.02; $MnSO_4 \cdot H_2O$, 0.06. Peptone and yeast extract were used as nitrogen sources in concentrations of 2.0 and 1.0 g/L, respectively. Peptone contained *c*. 18%, *w*/*w* nitrogen and *c*. 30%, *w*/*w* carbon, whereas yeast extract contained *c*. 7%, *w*/*w* nitrogen and *c*. 12%, *w*/*w* carbon. The initial pH for all media before and after sterilization (performed at $T = 115$ °C, 20 min) was 6.0 ± 0.1. In all performed trials, crude glycerol was employed as a microbial substrate. The feedstock was provided by the Hellenic biodiesel-producing

industry "ELIN VERD SA" (Velestino, Magnissia Prefecture, Greece). The purity of crude glycerol was as follows: glycerol content ≈88%, w/w, salts of potassium and sodium ≈6%, w/w, non-glycerol organic compounds (mostly free fatty acids) ≈1%, w/w and water (5%, w/w). In the first part ("screening" experiment), all strains were cultivated in shake-flask fermentations in media in which initial glycerol (Gly_0) concentration was adjusted to ≈50 g/L. The culture was performed under the limitation of nitrogen (initial C/N molar ratio ≈55 moles/moles) to direct the metabolic activity in favor of SCO production [2,5,6]. Two of the strains that presented better performances (namely the strains NRRL Y-27013 and NRRL Y-6985) were cultured at higher Gly_0 amounts (≈90 and ≈110 g/L), while the initial nitrogen quantity remained as before (therefore, peptone and yeast extract concentrations were 2.0 and 1.0 g/L, respectively); thus, in the second set of experiments, besides the increasing Gly_0 concentrations, the initial molar ratio C/N also increased (at $Gly_0 \approx 90$ g/L, the initial molar ratio C/N was ≈100 moles/moles, while at $Gly_0 \approx 110$ g/L, the initial molar ratio C/N was ≈120 moles/moles). The calculations took into consideration the purity of the industrial feedstock to yield the above-mentioned Gly_0 concentrations of the media.

Most of the submerged fermentations were conducted in non-baffled Erlenmeyer flasks of 250 mL filled at 20%, v/v of previously sterilized culture medium. The pre-cultures were prepared in similar 250 mL Erlenmeyer flasks, equally filled at 20%, v/v with previously sterilized YPD medium (glucose, peptone and yeast extract at 10 g/L of each), which were aseptically inoculated from a fresh slant that contained the strain. Pre-cultures were incubated in an orbital shaker (Zhicheng ZHWY 211C; PR of China) for 48 ± 4 h at 180 ± 5 rpm, T = 28 ± 1 °C. The concentration of yeast dry biomass of the pre-culture at 48 ± 4 h was 5.0 ± 0.5 g/L. Flasks of the principal cultures were inoculated with 1.0 mL of pre-culture and were incubated in the same shaker at the same conditions as the pre-cultures.

To assess the kinetics of the "red" yeasts on glycerol-based media, the contents of flask samples were periodically subjected to centrifugation (9000× g/15 min at T = 10 °C) in a Hettich Universal 320R (Vlotho, Germany) centrifuge. Recovered wet cells were extensively washed with distilled water and were re-centrifuged. Yeast dry biomass (X, g/L) was determined by means of its DCW wet biomass, which was put in a pre-weighed McCartney glass bottle and placed at T = 85 ± 2 °C until constant weight (in most cases for *c.* 30 ± 4 h). The pH of the culture medium was measured off-line using a Jenway 3020 (Cole-Parmer, Eaton Socon, UK) pH meter. In all the fermentations and irrespective of the strain or the Gly_0 concentrations, pH in the medium ranged between 5.1 and 5.9 and there was no need to perform correction. The dissolved oxygen tension (DOT) was measured off-line using a selective electrode (Bante Instruments Inc., Shanghai, China) according to Filippousi et al. [42]. The DOT was always ≥10% v/v during all growth phases for all shake-flask fermentations, providing evidence that all trials were performed under fully aerobic conditions [42].

Residual glycerol in the growth medium was quantitatively determined through HPLC analysis [40]. The free amino nitrogen (FAN) concentration in the liquid samples was determined according to Kachrimanidou et al. [43]. Total cellular lipids (L, g/L) were extracted from the microbial DCW with the modified protocol of Folch (placement of mixture of chloroform/methanol 2:1 v/v into the McCartney bottle containing the yeast DCW for at least 5 days, cell debris removal through filtration and solvent mixture evaporation), as presented in detail in the work of Sarantou et al. [44]. Cellular lipids were converted to their respective fatty acid (FA) methyl-esters that were subsequently analyzed in a gas chromatograph (GC-FID) apparatus (Fisons 8060, Markham, ON, Canada), equipped with a Chrompack (São Paulo, Brazil) column (60 m × 0.32 mm) and a flame ionization detector using helium as a carrier gas (2.0 mL/min), according to Giannakis et al. [45]. Finally, intra-cellular polysaccharides (*IPS*, g/L) were quantitatively determined according to the modified protocol adapted by Argyropoulos et al. [46].

To collect LFWs obtained from the microbial lipid fermentation processes, trials on glycerol ($Gly_0 \approx 50$ g/L) were conducted in shake flasks of a total volume of 2.5 L, filled with 450 mL of previously sterilized culture medium and inoculated with 50 mL of exponential pre-culture. These 2.5 L flasks were incubated in the same orbital shaker as before for 320 ± 10 h at 250 ± 10 rpm and T = 28 ± 1 °C. LFWs were collected through centrifugation (see above) of the cultures and stored in a freezer (T = 4 ± 1 °C). These fermentation wastewaters contained glycerol at a final concentration of 2.5 ± 0.5 g/L and a FAN concentration of 15 ± 5 mg/L. Phosphate and sulfate ions were also found in these LFWs.

2.2. Fungi

In this work, seven higher fungi (mushrooms) were used: *Pleurotus ostreatus* (AMRL 135), *P. eryngii* (AMRL 161), *P. pulmonarius* (AMRL 177), *Ganoderma applanatum* (AMRL 341), *G. resinaceum* (AMRL 325), *G. lucidum* (AMRL 330) and *Lentinula edodes* (AMRL 121), deriving from the Laboratory of Edible Fungi of Institute of Technology of Agricultural Products/Hellenic Agricultural Organization—Dimitra. The strains were maintained on potato dextrose agar (PDA, Merck, Germany) at T = 4 ± 1 °C and regularly sub-cultured. Prior to experiments, fresh mycelia were produced on PDA Petri dishes by incubation at T = 26 ± 1 °C and relative humidity of 75%.

2.3. Substrates for Fungal Growth

Agro-residues, i.e., olive crop residues (leaves and branches) (OC), rice husk (RH), beech wood shavings (BW), wheat straw (WS), wheat bran and poplar sawdust, originated from Greek farms and industries and coffee residue (CR) was obtained from local coffee shops. They were dried in a T = 60 ± 1 °C oven, until constant weight (Elvem, Athens, Greece) and milled to size <0.2 mm in a Janke & Kunkel, IKA-WERK, analytical mill (Staufen im Breisgau, Germany). Several physicochemical analyses were performed on the raw residues and the final substrates before their inoculation with the mushroom strains. Total nitrogen and organic matter were determined according to the Kjeldahl (Total Kjeldahl, Nitrogen, TKN) method (APHA) [47] and Sparks et al. [48], respectively. Protein content was determined through nitrogen concentration, with the use of the factor 6.25. The FAN concentration of LFW was assayed by the ninhydrin colorimetric method according to Lie [49]. Regarding the pH and electrical conductivity (EC) calculation, residue aliquots of 10 g were suspended in 100 mL distilled water for 2 h and the above parameters were measured using a Crison pH meter GLR 21 (Barcelona, Spain) and a Hanna Instruments HI 8733 (Padova, Italy) electrical conductivity instrument, respectively.

Prior to substrate preparation, dry WS, BW and OC residues were cut to ~2 cm using a cutting machine (Novital, mod. Magnum-4V, Lonate Pozzolo VA, Italy) and then they were (a) soaked in LFW from *R. toruloides* fungal culture or tap water for 5–8 h, with the excess water being drained off after 2 h, and (b) sprayed with 20%, w/w LFW. Different substrates were prepared by combining residues (dry substrate weight) as follows: (1) CR:80, WS:18 (named CW); (2) CR:80, CB:18 (CB); (3) OC:70, WS:12 (OW); (4) OC:70, BW:12 (OB); (5) RH:70, WS:12 (RW); and (6) RH:70, BW:12 (RB). Wheat bran and soybean flour were used as supplements to obtain a final C/N ratio of 20–30, CW and CB at 1%, w/w each and the other four substrates (OW, OB, RW and RB) at 13% and 5%, w/w, respectively. Calcium carbonate was also added to the substrates (1% w/w, in terms of dry weight) to obtain a pH = 6.0–7.5. Glass tubes (200 × 28 mm, five/substrate/strain) were uniformly filled with 80 mL of substrates and autoclaved at T = 121 ± 1 °C (1.1 atm) for 2 h. The water content of the sterilized substrates was 60–75%. When room temperature was reached, tubes were inoculated with one agar plug (of 6 mm diameter cut from the periphery of a fresh fungal colony grown on PDA) and finally, they were incubated at T = 26 ± 1 °C (in an ENTERLAB, mod. GROW-1300 HR, Terrassa, Spain) in the dark, until full colonization of the substrate.

2.4. Growth Rate Measurement and Glucosamine Content Determination

During substrate colonization by the fungi, the measurements of colony diameter were taken in two perpendicular directions of the tubes every two to three days and the mycelial growth rate (Kr, expressed in mm/d) was determined in five tubes [50]. At 100% of colonization, duplicate tube samples were used for biomass estimation. They were frozen at $T = -20 \pm 1$ °C for 48 h and then dried using a Heto LyoLab 3000 freeze-dryer (Heto-Holten Als, Denmark), milled and sieved. The measurement of N-acetylglucosamine, as derived from fungal chitin hydrolysis, was used for the indirect determination of biomass production [51]. Specifically, 2 g of dry sample (substrate and biomass) was mixed with 5 mL of 72% H_2SO_4 (Merck, Darmstadt, Germany) and agitated for 30 min at 130 ± 2 rpm in a rotary shaker (ZHWY-211C, Shanghai, China). Following dilution with 54 mL deionized water, the mixture was autoclaved for two hours at $T = 121 \pm 1$ °C to initiate hydrolysis. Using NaOH solution, the hydrolysate was neutralized. Equal volumes of (3 mL) of 5% (w/v) $NaNO_2$ and 5% $KHSO_4$ were added to a 3 mL sample from the previous step. The mixture was shaken for 15 min and then centrifuged at 1500× g (Micro 22R, Hettich, Germany), for 2 min at $T = 2 \pm 0.1$ °C. Following that, a 3 mL sample of the supernatant was mixed with 1 mL of 12.5% $NH_4SO_3NH_2$ and shaken for 5 min. Next, 1 mL of 0.5% 3-methyl-2-benzothiazolonehydrazone hydrochloride (MBTH) solution was added and heated for 3 min in a boiling water bath and then cooled, and 1 mL of 0.5% $FeCl_3$ was added. After 30 min, the absorbance was measured. The glucosamine was quantified spectrophotometrically at 650 nm using a Jasco V-530 UV/VIS spectrophotometer (Tokyo, Japan) and the results were expressed as mg of fungal biomass/g of dry substrate. Glucosamine standard curves were obtained using several concentrations of N-acetylglucosamine (Sigma-Aldrich, Taufkirchen, Germany). The glucosamine content of each fungus was determined through the mycelia produced in liquid cultures for 25 days, in 100 mL Erlenmeyer flasks with glucose (Alpha Aesar, Karsruhe, Germany) 30 g/L, yeast extract (Fluka, Steinheim, Germany) 3 g/L, peptone (Merck, Darmstadt, Germany) 3 g/L and $CaCO_3$ 0.1 g/L, as growth medium, at $T = 26 \pm 1$ °C under static conditions [26]. The equations correlating mycelial mass with glucosamine content, for each strain, were obtained after fitting the linear model to the experimental data.

2.5. Data Analysis

For all submerged fermentation cultures performed, each experimental point of all the kinetics presented in the tables and figures is the mean value of two independent determinations, in which two lots of independent cultures were conducted using different inocula. The standard error (SE) for most of the experimental points was ≤15%. Kinetics concerning the submerged lipid-production cultures were plotted using Kaleidagraph 4.0 Version 2005 showing the mean values with the standard error mean.

2.6. Abbreviations and Units

X: biomass (dry cell weight (DCW)) (g/L); L: lipids (g/L); *Gly*: glycerol (g/L); *IPS*: intra-cellular polysaccharides (g/L); r_{Gly}: glycerol consumption rate (g/L/h); $Y_{X/Gly}$: yield of total biomass produced on glycerol consumed (g/g); $Y_{L/Gly}$: yield of lipids produced on glycerol consumed (g/g); L/X: lipids in DCW (%, w/w); IPS/X: intra-cellular polysaccharides in DCW (%, w/w); *Kr*: mycelial growth rate (mm/d).

3. Results and Discussion

3.1. Screening of "Red" Yeasts for Lipid Production

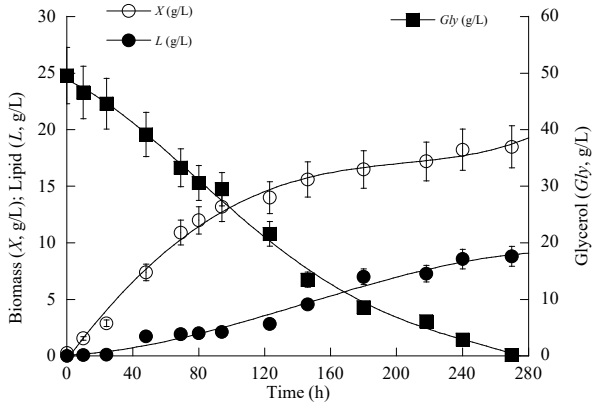
The four "red" yeast strains used in the present study (*R. kratochvilovae* FMCC Y-70, *R. toruloides* NRRL Y-27013, *R. toruloides* NRRL Y-17902 and *R. toruloides* NRRL Y-6985) were cultured in shake-flask experiments under nitrogen limitation (glycerol excess) with Gly_0 concentration ≈50 g/L (initial C/N molar ratio ≈55 moles/moles) and the obtained results are presented in Table 1. All the tested strains presented important quantities of consumed glycerol and varying concentrations of total yeast biomass, cellular lipids and

cellular polysaccharide production. In all fermentations performed, after a given point (i.e., at t ≈ 50 h), media were under limited nitrogen conditions (initial FAN = 80 ± 10 mg/L, FAN after t ≈ 50 h = 15 ± 5 mg/L). Three out of the four screened strains (namely NRRL Y-27013, NRRL Y-17902 and NRRL Y-6985) presented almost equivalent kinetic and physiological profiles; for these strains, the consumption rate of glycerol (r_{Gly}) as calculated by the formula $r_{Gly} = -\frac{\Delta Gly}{\Delta t}$ for the period, where glycerol was virtually found in non-negligible concentrations in the growth medium (i.e., $Gly \geq 5.0$ g/L), was unaffected by the nitrogen-limited conditions in the medium and was almost similar for all these trials (r_{Gly} = 0.20 ± 0.2 g/L/h), while at t = 240–270 h after inoculation, glycerol consumption was almost complete (at that time, the consumed glycerol represented *c.* 94–99%, *w*/*w* of the total initial substrate concentration). Moreover, for these strains, the values of total yeast DCW, *L*, *IPS*, *L*/*X* and *IPS*/*X* constantly increased, reaching their maximum values at the end of fermentation (viz. when almost all available glycerol quantity had been assimilated). On the other hand, a different profile in which *L*/*X* and *IPS*/*X* values presented a peak at the middle of fermentation and thereafter decreased, although the quantity of non-assimilated glycerol was high, was demonstrated for strain FMCC Y-70. The kinetics of the culture in one characteristic case (viz. the strain *R. toruloides* NRRL Y-6985) are illustrated in Figure 1. It is interesting to indicate that, as previously mentioned, the values of *L*/*X* and *IPS*/*X* increased simultaneously, suggesting that the microorganism accumulated reserve lipid and reserve non-lipid compounds at the same time. This agrees with the results presented for *R. toruloides* CBS 14 growing on glucose under nitrogen-limited conditions [52,53]. On the contrary, for other strains (i.e., strains NRRL Y-27012 and DSM 4444), the *IPS*/*X* values were high in the first culture phases, and in the presence of assimilable nitrogen in the medium, they deceased as the fermentation proceeded, with a simultaneous increase in the *L*/*X* values [13,54].

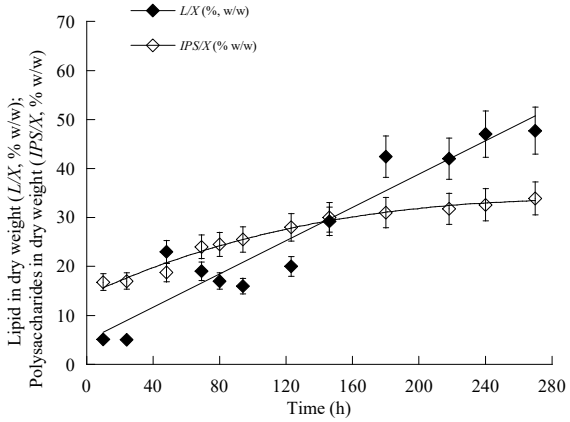
Table 1. Quantitative data of *R. kratochvilovae* FMCC Y-70, *R. toruloides* Y-17902, *R. toruloides* NRRL Y-27013 and *R. toruloides* NRRL Y-6985 strains deriving from kinetics on crude glycerol, in nitrogen-limited shake-flask cultures, with initial glycerol ($Glyc_0$) concentration ≈ 50 g/L and initial nitrogenous compounds peptone and yeast extract added at 2.0 and 1.0 g/L (initial molar ratio C/N ≈ 55 moles/moles). Four different points in the fermentations are represented: (1) when the maximum quantity of total dry yeast biomass (*X*, g/L) was observed; (2) when the maximum quantity of lipids per DCW (*L*/*X*, % *w*/*w*) was observed; (3) when the maximum quantity of intra-cellular polysaccharides per DCW (*IPS*/*X*, % *w*/*w*) was observed; (4) when the maximum quantity of absolute lipid value (L, g/L) was observed. Culture condition: initial glycerol concentration (Glyc_0 ≈ 50 g/L), growth on 250 mL conical flasks at 180 ± 5 rpm, initial pH= 6.0 ± 0.1, culture pH ranging between 5.1 and 5.9, incubation temperature *T* = 28 ± 1 °C. Each experimental point is the mean value of two measurements (SE for most experimental points is ≤17%).

Strains		**Time (h)**	$Glyc_{\mathrm{cons}}$ **(g/L)**	***X*** **(g/L)**	$Y_{\mathrm{X/Gly}}$ **(g/g)**	***L*** **(g/L)**	***L*/*X*** **(%, *w*/*w*)**	***IPS*/*X*** **(%, *w*/*w*)**
NRRL Y-27013	1, 2, 3, 4	240	47.7 ± 1.9	19.0 ± 2.0	0.40	8.8 ± 0.9	46.3	31.2
NRRL Y-6985	1, 2, 3, 4	270	49.4 ± 1.7	18.5 ± 1.6	0.37	8.8 ± 0.8	47.6	33.9
NRRL Y-17902	1, 2, 3, 4	250	46.7 ± 1.9	19.0 ± 2.2	0.41	8.6 ± 1.0	45.2	33.1
FMCC Y-70	2, 3, 4	82	21.2 ± 2.2	9.1 ± 1.5	0.43	2.3 ± 0.5	25.4	39.9
	1	245	43.7 ± 2.7	15.1 ± 1.3	0.35	1.8 ± 0.4	12.2	32.1

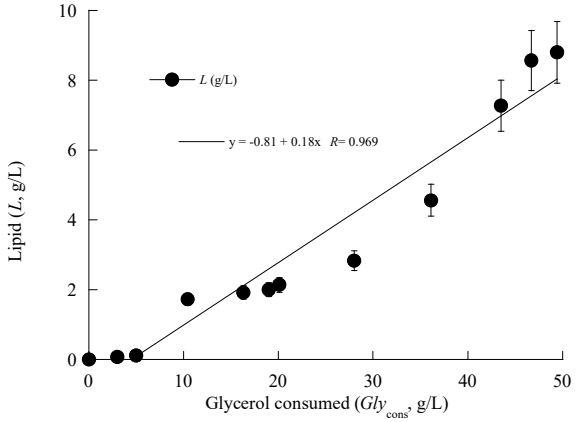
Fermentation time (h); yield of total yeast biomass produced on glycerol consumed (YX/Gly, g/g); and quantities of total dry yeast biomass (*X*, g/L), total lipids (*L*, g/L) and glycerol consumed ($\mathrm{Glyc}_{\mathrm{cons}}$, g/L) are also depicted for all the above-mentioned fermentation points.



(**a**)



(**b**)



(**c**)

Figure 1. Changes in total biomass (*X*, g/L), glycerol (*Gly*, g/L) and cellular lipids (*L*, g/L) (**a**) and cellular lipid per DCW (*L*/*X*, % *w*/*w*) and cellular polysaccharides per DCW (*IPS*/*X*, % *w*/*w*) (**b**) as a function of fermentation time for *R. toruloides* NRRL Y-6985 grown on glycerol in shake-flask trials. Representation of global conversion yield of lipids produced per unit of glucose consumed as shown by linear regression of produced lipids as a function of consumed glycerol (**c**) for the same set of data. Each point is the mean value of two independent measurements. SE $\leq$ 15%.

The fatty acid (FA) composition of the cellular lipids synthesized in this set of cultures is presented in Table 2 (concerning t from 120 to 160 h after inoculation). The principal cellular FAs detected were mostly oleic acid (Δ9C18:1) and palmitic acid (C16:0). Cellular lipids of the yeast *R. toruloides* NRRL Y-27013 contained increased concentrations of saturated FAs (i.e., C16:0 = 39.9%, *w*/*w* and C18:0 = 13.5%, *w*/*w*, meaning that the concentration of saturated FAs was = 53.4%, *w*/*w* of total cellular lipids produced). In fact, when the concentration of saturated cellular FAs is ≥40%, *w*/*w* of total cellular lipids, as was the case of the mentioned strain NRRL Y-27013, the yeast produces lipids of a similar composition to palm oil [55–57]. Palm oil has an important number of applications, including food and biodiesel production, and its enormous utilization, specifically during the last decade, is a leading contributor to tropical deforestation, the major result of which is the constantly increasing CO_2 emissions that have been noted [55]. Nevertheless, all yeast lipids synthesized (see Table 2) contained high concentrations of oleic acid; therefore, these yeast SCOs can be used for the synthesis of second-generation biodiesel [2,5,6,56]. Finally, poly-unsaturated cellular FAs (specifically the ones with ≥3 double bonds) were detected in low or negligible quantities in the cellular lipids of the tested yeasts, in accordance with the literature [2,3,6,58,59].

Table 2. Fatty acid composition of the cellular lipids produced by yeast strains cultivated on crude glycerol in shake-flask experiments ($Glyc_0$ ≈ 50 g/L, C/N ≈ 55 moles/moles). Time of fermentation for the determination of the fatty acid composition was between 120 and 160 h after inoculation. Culture conditions were as in Table 1.

Strain/Fatty Acid	C16:0 (%, *w*/*w*)	C18:0 (%, *w*/*w*)	C18:1 (%, *w*/*w*)	C18:2 (%, *w*/*w*)	C18:3 (%, *w*/*w*)
NRRL Y-27013	39.9	13.5	42.8	1.6	n.d.
NRRL Y-6985	30.1	2.6	54.2	2.0	0.8
NRRL Y-17902	31.2	2.7	54.0	1.3	0.7
FMCC Y-70	25.9	9.5	39.5	15.5	7.9

n.d.: <0.5%, *w*/*w*.

3.2. Trials in Higher Initial Glycerol Concentrations and C/N Molar Ratios

Two of the four previously tested "red" yeast strains (*R. toruloides* NRRL Y-27013 and *R. toruloides* NRRL Y-6985) were cultured in shake-flask experiments under higher Gly_0 concentrations (≈90 g/L and ≈110 g/L), while the initial nitrogen amount remained the same as before (*viz*. peptone and yeast extract at 2.0 and 1.0 g/L, respectively). Therefore, in the second set of experiments, besides the increasing Gly_0 concentrations, the initial molar ratio C/N significantly increased (at Gly_0 ≈ 90 g/L, the initial C/N molar ratio was ≈100 moles/moles, while at Gly_0 ≈ 110 g/L, it was ≈120 moles/moles). The obtained results as regards *R. toruloides* NRRL Y-27013 and *R. toruloides* NRRL Y-6985 are shown in Table 3. From the obtained results, it can be indicated that for both strains and despite both the elevated Gly_0 concentrations and initial molar ratios C/N imposed, high quantities of glycerol had been assimilated. For all trials, as previously indicated, the r_{Gly} calculated by the formula $r_{Gly} = -\frac{\Delta Gly}{\Delta t}$ was ≈0.26–0.27 g/L/h, which was higher than that of the previous experiment with the lower Gly_0 concentration and initial molar ratio C/N imposed. It is also interesting to indicate that the fermentation was stopped, however, the trend of the kinetics for all experiments was that the available glycerol quantity would be finally grosso modo consumed; therefore, even higher quantities of DCW and intra-cellular metabolites could have been synthesized. For both studied strains (*viz*. NRRL Y-27013 and NRRL Y-6985), lipid and polysaccharide quantities (in terms of L/X and IPS/X, in %, *w*/*w*) globally increased. In absolute values, the significant lipid quantity of 12.6 g/L was recorded for both strains in the cultures with the increased Gly_0 concentration and initial molar ratio C/N imposed. However, by comparing the L/X values for all the performed trials and

for both the strains NRRL Y-27013 and NRRL Y-6985, it can be seen that the more the Gly_0 concentration and the initial C/N molar ratio increased, the lower the recorded L/X values were (see Tables 1 and 3). Therefore, although lipid quantities increased in absolute values, lipids in DCW values decreased, presumably in favor of the biosynthesis and accumulation of intra-cellular polysaccharides, the IPS/X values of which drastically increased (see Tables 1 and 3). Once more, therefore, it can be suggested that the studied strains (NRRL Y-27013 and NRRL Y-6985) presented a biochemical similarity with the strain CBS 14 growing on glucose, where the values of IPS/X and L/X constantly increased in response to the nitrogen-limited conditions imposed [52,53]. It is known that after nitrogen depletion in the medium, a fast reduction of the concentration of cellular AMP occurs [1,2]. As a response, there will subsequently be either a decrease in the activity of the Krebs cycle (decrease in the activity of NAD^+ or $NADP^+$ isocitrate dehydrogenase, leading to the enhanced synthesis of cellular fatty acids and triacylglycerols) or a decrease in the glycolysis rate due to decreased activity of phospho-fructokinase (for reviews, see [1,2,53,59]). Apparently, for the case of both strains NRRL Y-27013 and NRRL Y-6985 (and potentially for the strain NRRL Y-17902), the response to the imposed nitrogen limitation seems to be the accumulation of both lipids and storage polysaccharides. On the other hand, some substrate inhibition was observed against both NRRL Y-27013 and NRRL Y-6985 strains, due to the increased Gly_0 concentration imposed, as calculated through the biomass conversion yield $Y_{X/Gly}$ obtained in all fermentations. Therefore, the yield $Y_{X/Gly}$ calculated by the formula $Y_{X/Gly} = \frac{X_{max}-X_0}{Gly_{cons}}$ was ≈ 0.40 g/g for the strain NRRL Y-27013 at Gly_0 ≈ 50 g/L and decreased to ≈0.32–0.33 g/g at the highest initial glycerol concentrations. Concerning the strain NRRL Y-6985, at Gly_0 ≈ 50 g/L, the yield value was ≈0.38 g/g, decreasing to ≈0.32 g/g at the highest Gly_0 concentration. Representative results of DCW production and lipids in DCW values for yeasts cultivated on glycerol and the comparisons with the current study are demonstrated in Table 4.

Table 3. Quantitative data of *R. toruloides* NRRL Y-27013 and *R. toruloides* NRRL Y-6985 strains deriving from kinetics on crude glycerol, in nitrogen-limited shake-flask cultures, with increasing initial glycerol (Gly_0) concentration (≈90 g/L, C/N ≈ 100 moles/moles; ≈110 g/L, C/N ≈ 120 moles/moles) at constant initial nitrogen concentration in the medium (initial nitrogenous compounds were peptone and yeast extract added at 2.0 and 1.0 g/L, respectively). Four different points in the fermentations are represented: (1) when the maximum quantity of total dry yeast biomass (X, g/L) was observed; (2) when the maximum quantity of lipids per DCW (L/X, % w/w) was observed; (3) when the maximum quantity of intra-cellular polysaccharides per DCW (IPS/X, % w/w) was observed; (4) when the maximum quantity of absolute lipid value (Ls, g/L) was observed. Fermentation time (h); quantities of yeast biomass (X, g/L), total lipids (Ls, g/L) and glycerol consumed ($Glyc_{cons}$, g/L); and yield of total biomass produced per glycerol consumed ($Y_{X/Gly}$, g/g) are also depicted for all the above-mentioned fermentation points. Culture conditions as in Table 1.

$Glyc_0$ (g/L)		Time (h)	$Glyc_{cons}$ (g/L)	X (g/L)	$Y_{X/S}$ (g/g)	L (g/L)	L/X (%, w/w)	IPS/X (%, w/w)
R. toruloides NRRL Y-27013								
≈90	1, 2, 3, 4	264	70.4 ± 3.1	23.4 ± 2.7	0.33	11.1 ± 1.6	47.4	34.9
≈110	1, 2, 3, 4	336	89.0 ± 2.9	29.3 ± 3.3	0.33	12.6 ± 1.9	43.0	41.3
R. toruloides NRRL Y-6985								
$Glyc_0$ (g/L)		Time (h)	$Glyc_{cons}$ (g/L)	X (g/L)	$Y_{X/S}$ (g/g)	L (g/L)	L/X (%, w/w)	IPS/X (%, w/w)
≈90	1, 2, 3, 4	288	77.1 ± 2.1	24.4 ± 2.9	0.32	11.0 ± 1.9	45.1	36.2
≈110	1, 2, 3, 4	336	90.9 ± 3.3	29.1 ± 2.6	0.32	12.6 ± 2.7	43.2	42.1

Table 4. Representative results concerning the production of total dry biomass and lipids in DCW values by yeast species cultivated on glycerol under various fermentation modes.

Strain	Cultivation Type	*X* (g/L)	*L*/*X* (%, *w*/*w*)	Reference
Cryptococcus curvatus ATCC 20509	Fed-batch reactor	118.0	25.0	Meesters et al. [60]
C. curvatus ATCC 20509	Fed-batch reactor	32.9	52.9	Liang et al. [61]
C. curvatus ATCC 20509	Fed-batch reactor	22.0	49.0	Cui et al. [62]
Yarrowia lipolytica MUCL 28849	Fed-batch reactor	42.2	38.2	Fontanille et al. [63]
Rodosporidium toruloides AS2.1389	Batch reactor	26.7	69.5	Xu et al. [64]
R. toruloides Y4	Batch reactor	35.3	46.0	Uçkun Kiran et al. [65]
R. toruloides Y4	Batch flasks	24.9	48.9	Yang et al. [66]
Lipomyces starkeyi DSM 70296	Batch flasks	34.4	35.9	Tchakouteu et al. [67]
R. toruloides DSM 4444	Fed-batch reactor	37.4	51.3	Leiva-Candia et al. [68]
R. toruloides DSM 4444	Fed-batch reactor	41.0	60.0	Signori et al. [69]
R. toruloides DSM 4444	Batch flasks	37.0	37.0	Papanikolaou et al. [70]
R. toruloides ATCC 10788	Batch flasks	10.3	34.0	Uprety et al. [71]
R. toruloides AS 2.1389	Batch flasks	18.9	64.5	Kamal et al. [72]
R. toruloides DSM 4444	Batch flasks	28.9	43.3	Diamantopoulou et al. [13]
R. toruloides DSM 4444	Batch flasks	27.3	54.6	Sarantou et al. [44]
C. curvatus ATCC 20509	Batch flasks	12.6	48.4	Karayannis et al. [7]
R. toruloides NRRL Y-27013	Batch flasks	29.3	43.0	Present study
R. toruloides NRRL Y-6985	Batch flasks	29.1	43.2	Present study

The FA composition of the cellular lipids synthesized in this set of cultures is presented in Table 5 (analysis performed at t ranging between 120 and 160 h after inoculation). As in the previous set of experiments, the principal cellular FAs were mostly the C18:1 and the C16:0. As compared to the trial performed at $Gly_0 \approx 50$ g/L, the cellular lipids of the strain NRRL Y-27013 in the trials with higher FA Gly_0 concentrations imposed seemed less saturated (see Tables 2 and 5). In contrast, for the strain NRRL Y-6985, no significant differences were recorded in the trials with the various Gly_0 concentrations imposed. In all cases, cellular C18:1 was the principal cellular FA, rendering the lipids of *R. toruloides* as precursors for the production of second-generation biodiesel [2,5,6,56].

Table 5. Fatty acid composition of the cellular lipids produced by the selected yeast strains NRRL Y-27013 and NRRL Y-6985 cultivated on crude glycerol in shake-flask experiments ($Glyc_0 \approx 90$ g/L and $Glyc_0 \approx 110$ g/L). Time of fermentation for the determination of the fatty acid composition was between 120 and 160 h after inoculation. Culture conditions were as in Table 1.

Gly_0 (g/L)	Strain	C14:0 (%, *w*/*w*)	C16:0 (%, *w*/*w*)	C16:1 (%, *w*/*w*)	C18:0 (%, *w*/*w*)	C18:1 (%, *w*/*w*)	C18:2 (%, *w*/*w*)	C18:3 (%, *w*/*w*)
≈90	NRRL Y-27013	2.1	25.3	n.d. *	11.1	51.5	6.7	3.3
	NRRL Y-6985	n.d.	28.8	11.9	2.1	54.8	2.1	0.3
≈110	NRRL Y-27013	2.1	26.3	n.d.	11.0	49.8	5.4	5.4
	NRRL Y-6985	n.d.	27.0	10.9	3.1	55.2	3.1	0.7

* n.d.: <0.5%, *w*/*w*.

3.3. Substrate Analysis for Mushroom Cultivation

Primary analysis conducted on the residues used in the present study concerning their pH, EC and N and C contents provided the basic information needed before their mixture and final substrate synthesis (Table 6). The six substrate formulations prepared contained different amounts of residues so that the appropriate conditions (e.g., C/N = 20–30) and the necessary ingredients in adequate quantities for fungal growth were provided [9]. Parame-

ters such as total C, N, C/N ratio, pH and EC of the final substrates (before inoculation) are important for mycelium growth and mushroom fructification.

Table 6. Physicochemical profile of agro-industrial residues used in the study.

Residue	Moisture Content (%)	pH	EC (μS/cm)	Nitrogen (% d.w.)	Protein (% d.w.)	Carbon (% d.w.)
WS *	7.15	7.35	840	0.65	4.06	31.44
BW	7.89	5.57	285	0.23	1.44	33.07
CR	7.20	5.36	228	1.90	11.88	32.83
OC	6.15	5.24	516	1.24	7.75	39.00
RH	7.92	6.84	465	0.35	2.19	28.52
WB	10.86	6.21	894	3.37	21.06	31.79
SF	10.58	6.06	752	5.88	36.75	67.59

Data are presented as mean values from duplicated measurements. * WS: wheat straw, BW: beech wood shavings, CR: coffee residue, OC: olive crop, RH: rice husk, WB: wheat bran, SF: soybean flour.

The results show (Table 7) that the C (%) concentration among the different substrates presented no great differences, as values ranged from 31.25 to 38.70% (*w*/*w*). The C/N ratio varied from 19.03 (CW) to 31.46 (RB), the desirable values for mushroom production [26,27]. The pH values ranged from 6.28 to 6.94, making *Pleurotus* cultivation feasible, as the fungal mycelium obtains nutrients from the substrate at a particular range of pH, whereas rapid mycelial growth occurs at pH 6.4–7.8 [73,74]. The EC values presented great variations among substrates. The highest value was recorded in RB substrate treated with tap water (987 μS/cm, 0.987 mS/cm), and the lowest values were recorded in CW (229 μS/cm, 0.229 mS/cm). In general, the optimal EC for mushroom cultivation is usually in the range of 0.5 to 2.5 mS/cm. However, each species of mushrooms may have different requirements, as the optimal EC for the cultivation of the oyster mushroom (*P. ostreatus*) has been recorded at around 1.0 to 2.0 mS/cm, while for shiitake mushrooms (*L. edodes*), an EC of 1.5 to 2.5 mS/cm has been recommended [16].

Table 7. Composition and physicochemical profile of substrates used in solid-state fermentation experiments (final mixtures before inoculation).

	Substrate	C/N	Moisture Content (%)	pH	EC (μS/cm)	Protein (% d.w.)	C (% d.w.)	N (% d.w.)
H_2O	CW *	19.03	66.10	6.62	229	10.81	32.92	1.73
	CB	20.08	60.88	6.75	714	10.31	33.21	1.65
	OW	23.00	71.38	6.61	698	10.50	38.50	1.68
	OB	23.96	69.45	6.48	795	10.19	38.70	1.63
	RW	29.54	66.34	6.45	957	6.63	31.25	1.06
	RB	31.46	68.14	6.81	987	6.25	31.44	1.00
LFW (so) **	CW	19.03	62.73	6.28	266	10.81	32.92	1.73
	CB	20.08	58.97	6.87	721	10.31	33.21	1.65
	OW	23.00	68.58	6.66	741	10.50	38.50	1.68
	OB	23.96	70.12	6.48	658	10.19	38.70	1.63
	RW	29.54	72.90	6.94	495	6.63	31.25	1.06
	RB	31.46	74.45	6.57	321	6.25	31.44	1.00
LFW (sp)	CW	19.03	64.71	6.52	248	10.81	32.92	1.73
	CB	20.08	57.74	6.41	787	10.31	33.21	1.65
	OW	23.00	70.84	6.71	753	10.50	38.50	1.68
	OB	23.96	69.84	6.47	823	10.19	38.70	1.63
	RW	29.54	74.45	6.53	776	6.63	31.25	1.06
	RB	31.46	71.14	6.54	793	6.25	31.44	1.00

* CW: coffee residue + wheat straw, CB: coffee residue + beech wood shavings, OW: olive crop + wheat straw, OB: olive crop + beech wood shavings, RW: rice husk + wheat straw, RB: rice husk + beech wood shavings. ** LFW (so): soaked in lipid fermentation wastewater, LFW (sp): sprayed with lipid fermentation wastewater.

3.4. Mycelial Growth Rate

It is well known that mycelial growth and biomass production require the utilization of competent species and effective substrates [34,35]. Also, the combination of different residues leads to substrates with a more balanced chemical composition than the homogeneous ones, boosting quick development and high yields [75,76]. Thus, in our experiments, CR, OC, RH, BS and WS residues were used in several quantities for each substrate to achieve a C/N ratio of 20–30 and enhance their production [9] (see Table 7). In this study, *P. ostreatus*, *P. eryngii*, *P. pulmonarius*, *G. applanatum*, *G. resinaceum*, *G. lucidum* and *L. edodes* species were subjected to trials in which their ability to grow on the above wastes was evaluated, and in the view of water economy, two wetting methods were used: soaking and spaying substrates with LFWs.

The results of the mycelium linear growth (Kr, mm/d) for each substrate are shown in Figure 2. The colonization rates were higher in most substrates treated with LFWs compared to the control (tap water) (Figure 3), and they were affected by the substrate and the mushroom genus, as has been already demonstrated by previous studies [11,26,27,77]. The novel (non-conventional) substrates consisting of RH, OC and CR successfully supported mycelial growth, and Kr values varied among mushroom strains. Several of the highest growth rates (Kr > 6 mm/d) were recorded in RW (for *P. eryngii*, *G. resinaceum* and *L. edodes*) and RB (for *P. eryngii*, *G. resinaceum*) and in some cases, OW and OB gave better results (e.g., *P. pulmonarius* Kr = 5.46 mm/d in OW, *G. lucidum* Kr = 5.07 mm/d in OW and *G. applanatum* Kr = 6.36 mm/d in OB). Furthermore, *Ganoderma* species demonstrated fast colonization on OW and OB substrates, as well as high growth rates on RW and RB, probably due to the higher C/N ratio of these substrates and the relatively lower nitrogen content that acts beneficially. It seems, therefore, that the C/N ratio of the substrates was positively correlated with the growth rate. This positive correlation has been also previously reported by D'Agostini et al. [78] for *P. ostreatus* and *L. edodes*. Melanouri et al. [27] and Philippoussis et al. [9] observed higher growth rates (mean Kr = 9.58–6.92 mm/d) for *Pleurotus* spp. in a variety of substrates with C/N = 20–30, whereas in the study of Economou et al. [51], spent mushroom substrate of *P. ostreatus* (C/N = 30) favored the mycelial growth rate of *P. ostreatus* and *P. pulmonarius* (7–8 mm/day). However, in this study, OW and OB were unsuitable substrates for *P. eryngii* growth (Kr = 1.65 mm/d). Overall, as Kr data revealed that the addition of LFW had a slightly positive effect or no effect on the colonization of the various substrates tested by the several mushroom strains, it seems feasible to further use this waste in mushroom cultivation.

3.5. Mycelial Mass Production

As stated before, the parameter Kr expresses the hyphal progression on a substrate [79], but not the fungal ability to produce mycelial mass, so in the present study, the concentration of the biomass produced at the end of the colonization stage was estimated by glucosamine content measurement. The equations correlating mycelial mass with glucosamine content for each strain are presented in Table 8. The results showed that the highest biomass production was produced on substrates with the addition of LFW (soaked or sprayed) (Figure 3), probably because excess nitrogen, lipids and several minerals in the medium enhanced biomass production and RW and CR were the substrates where the production of biomass showed a significant increase; e.g., among the coffee substrates, these sprayed with LFW had a notable effect on *P. eryngii*. Particularly, LFW was beneficial to the growth of *Pleurotus* species, as *P. ostreatus* produced a biomass of more than 240 mg/g d.w. of substrate in all the treatments (maximum at RW: 443 mg/g d.w.; CW: 374 mg/g d.w.), while biomass production of *P. eryngii* and *P. pulmonarius* ranged from 186 to 292 mg/g d.w., with the highest values being recorded on OW and OB substrates. The addition of WS to the main substrate was more beneficial for biomass production than the addition of BW, as shown in *P. ostreatus* on CR and RH (soaked with LFW). Also, there was a significant positive effect of spaying LFW in the case of *L. edodes* (maximum at RB: 238 mg/g d.w.). In the current study, *Ganoderma* species presented lower biomass production in CW, CB,

OW and OB than in RW and RB substrates. The data also showed that the spaying method produced a higher biomass amount for *G. resinaceum* (182–292 mg/g, d.w.) compared to *G. lucidum* and *G. applanatum* in these substrates. It is worth noting that the addition of BW in CR resulted in higher values of biomass for *G. lucidum*. Contrariwise, in RW and RB, *G. resinaceum* and *G. lucidum* achieved twofold biomass production values (443–636 mg/g d.w.). Several studies in the past included data on mycelium mass concentration produced on SSFs. For example, Melanouri et al. [27] demonstrated a higher biomass production for *P. eryngii* and *P. ostreatus* grown in substrates containing CR (over 250 mg/g d.w.), but in RH, the biomass production was lower. Dedousi et al. [11] also recorded lower values of *P. ostreatus* biomass in RH substrate, in contrast with this study. However, the high mycelial mass production on grape pomace residue by *P. ostreatus* AMRL 135 (420 mg/g d.w.) shown by Papadaki et al. [35] was like the biomass produced by the same strain on RW in this study. Economou et al. [51] found that with an increase in the C/N ratio, biomass production decreased during SSF of waste mushroom substrate (spent WS), but in our case, with an increase in the C/N ratio, biomass production was enhanced. Regarding biomass production and mycelial growth rate, no correlation between these parameters could be established by examining the present data, although in other studies it has been reported that they are negatively related [27,51,80,81]. Finally, the addition of LWF was shown to have a positive effect on the biomass production of the examined fungi and CR and RH are better than OC in supporting the growth of *Pleurotus* spp., *Ganoderma* spp. and *L. edodes* mushrooms.

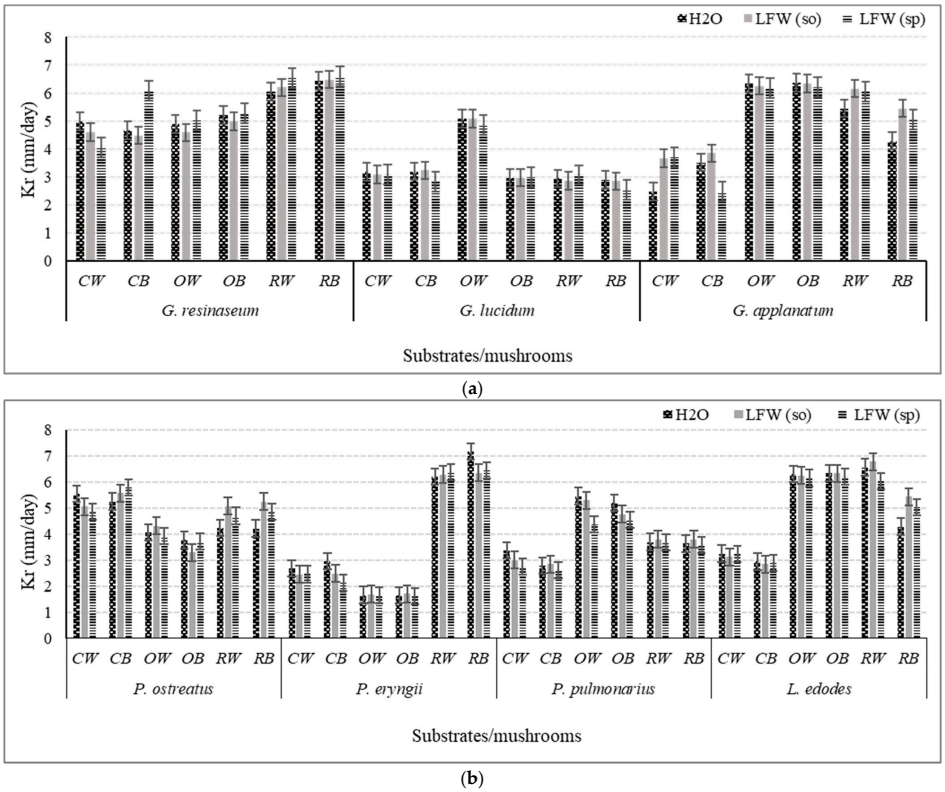


Figure 2. Mycelial growth rates (Kr, mm/d) of *P. ostreatus*, *P. eryngii*, *P. pulmonarius*, *G. applanatum*, *G. resinaceum*, *G. lucidum* and *L. edodes* mushrooms during solid-state fermentation (incubation—colonization stage) in substrates CW (coffee residue + wheat straw), CB (coffee residue + beech wood shavings), OW (olive crop + wheat straw), OB (olive crop + beech wood shavings), RW (rice husk + wheat straw) and RB (rice husk + beech wood shavings) prepared after soaking in lipid fermentation wastewater from *R. toruloides* (LFW) fungal culture or tap water or being sprayed with 20% *w*/*w* of LFW at 26 ± 1 °C. Mean values with error bars indicate the standard deviations from duplicate experiments of four replicates.

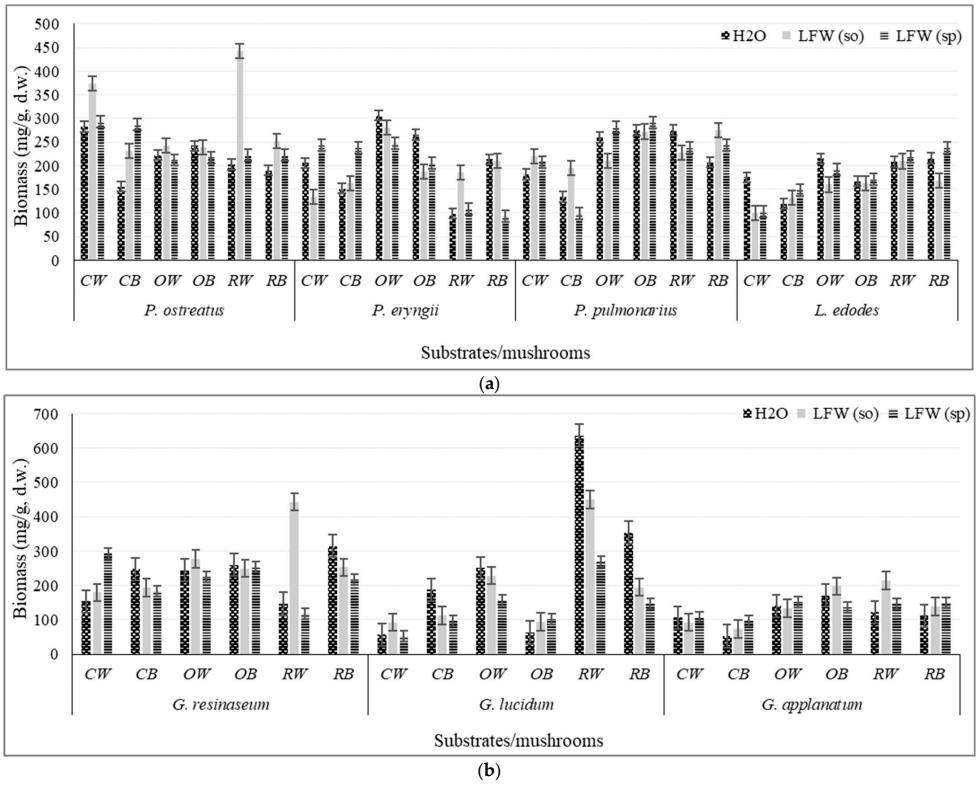


Figure 3. Biomass production (mg/g d.w. substrate) of *P. ostreatus*, *P. eryngii*, *P. pulmonarius*, *G. applanatum*, *G. resinaceum*, *G. lucidum* and *L. edodes* at the final stage of solid-state fermentation (incubation—colonization stage) in substrates CW, CB, OW, OB, RW and RB prepared after soaking in or being sprayed with LFW or tap water at 26 ± 1 °C. Mean values with error bars indicate the standard deviations from duplicate experiments of four replicates.

Table 8. Linear regression equations of glucosamine (mg) and biomass (g) of *Pleurotus*, *Ganoderma* and *Lentinula* species grown on liquid cultures with glucose as main carbon source under static conditions.

Fungi	Equation of Glucosamine mg (x)/Biomass g (y)	R^2
P. ostreatus	y = 0.0338x − 0.004	0.975
P. eryngii	y = 0.0324x − 0.038	0.993
P. pulmonarius	y = 0.0509x − 0.022	0.999
G. applanatum	y = 0.0168x − 0.009	0.947
G. lucidum	y = 0.0467x − 0.031	0.992
G. resinaceum	y = 0.0338x − 0.004	0.994
L. edodes	y = 0.0594x − 0.049	0.987

4. Conclusions

Crude glycerol, a by-product of the biodiesel production process, was revealed as a competitive substrate used in the lipid production process by novel *R. toruloides* strains. Cultures at high initial glycerol concentrations and C/N ratios were accompanied by significant biomass and lipid production. Due to the imposed nitrogen limitation, values of lipids and IPS (in DCW) increased with the fermentation time. Cellular lipids contained high concentrations of oleic acid and constituted ideal precursors for the synthesis of second-generation biodiesel. The use of LFWs, as replacements for tap water, in solid-state mushroom cultures where non-conventional substrates were used, proved to be suitable and beneficial for mushroom growth, especially when using the alternative spraying method. These results are in line with the circular economy principles of waste and water reuse, offering new options for environmental protection. Nevertheless, to complete the

investigation, further experiments concerning the impact of these new substrates on the quantity and quality of harvested mushrooms are taking place.

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