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Biotechnological Conversions of Mizithra Second Cheese Whey by Wild-Type Non-Conventional Yeast Strains: Production of Yeast Cell Biomass, Single-Cell Oil and Polysaccharides

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Abstract: The cultivation of oleaginous yeasts on various agro-industrial residues and the subsequent production of microbial lipids (single-cell oils), which can be used as starting materials for the synthesis of both "second generation" biodiesel and various types of oleochemicals, is considered as one of the most important approaches of the Industrial Biotechnology, in terms of circular economy and green and sustainable development. In this study, seven wild-type non-conventional yeast strains were evaluated for their growth on a semi-defined medium with cheese whey lactose as a sole carbon source. Five of these strains were further batch-cultivated into the complex substrate that contained second cheese whey, which is the main by-product of Mizithra whey cheese manufacture, after centrifugation and filtration without any extra mineral salts, carbon, or nitrogen source addition. All these five strains grown in second cheese whey produced mainly yeast biomass and to lesser extent microbial lipids and other interesting metabolites, such as polysaccharides. The strain *Cryptococcus curvatus* ATCC 20509 produced the highest total dry weight (TDW) amount (22.4 g/L), that contained 3.4 g/L of intra-cellular lipids, followed by *C. curvatus* NRRL Y-1511, which produced 20.6 g/L of TDW and 3.2 g/L lipids. A novel, non-previously systematically studied strain, namely *Papiliotrema laurentii* NRRL Y-2536, produced significant quantities of TDW (22.0 g/L) and, interestingly, secreted quantities of exopolysaccharides. Fed-batch shake-flask cultivation of *C. curvatus* ATCC 20509 in pretreated second cheese whey, pulse-supplemented with condensed cheese whey-derived lactose, led to the significant TDW quantity of 38.1 g/L that contained c. 57% *w*/*w* of total lipids (lipids at a concentration 21.7 g/L were produced). Cellular lipids of all microorganisms, mainly stored as triacylglycerols, contained in variable quantities the fatty acids $^{\Delta 9}$C18:1, C16:0, $^{\Delta 9,12}$C18:2 and C18:0, constituting perfect candidates for the synthesis of "second generation" biodiesel.

Keywords: cheese whey; oleaginous yeasts; single cell oil; "2nd generation" biodiesel; circular economy

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1. Introduction

The rapid increase in waste generation worldwide, according to the World Bank Group (global waste generation is expected to grow to 3.4 billion t. per year, a 70% increase compared to 2016 levels) [1] forces the scientific community and the society to find sustainable solutions, primarily reducing the volume of the produced residues and, consequently, utilizing these materials under eco-friendly processes. The reduction of wastes, combined with their valorization for production of new added-value products, benefits both the environment and the industry [2,3]. The majority of solid or semi-solid byproducts' streams produced by virtue of several agro-industrial activities (mostly we refer to the

food-production and food-processing sector residues) is currently utilized as animal feed, fertilizers, and substrates for composting [3]. Moreover, in several other cases, an important number of food-processing residues is directly landfilled, burnt, or discarded untreated in the aqueous environments (in the latter case, we refer to the liquid waste streams) with evident very important negative impact on the ecosystem [2], whereas c. 33% of all produced foodstuffs, totaling about 1.3×10^3 million t. per annum, is completely wasted around the world (Food and Agriculture Organization of the United Nations 2019 report; see: [3]). From all the above-mentioned analysis, therefore, it is evident that all aspects dealing with food waste valorization processes are widely studied, but unfortunately, many of the provided solutions are not yet applied by the majority of the implicated industries. Therefore, the continuously increasing food waste accumulation connected with the potential exhaustion of conventional fuels and the various resulting environmental issues (increase of CO_2 emission, global warming, climate change, eutrophication, etc.) [2] makes it increasingly necessary for industries to apply sustainable utilization of by-products and, consequently, to enter in the "meta-industry" era. Meta-industry, for the first-time reported as a term, describes the transition from classical industrial practice of product manufacture and waste disposal to an innovative next-generation industry, in which the by-product valorization would be integral part of industrial practice, in terms of circular bioeconomy ("metá" in ancient (and modern) Greek means "after"; therefore "meta-industry" would refer to the industrial applications after the synthesis of the principal industrial product, viz. to the industrial applications in the waste and residue streams generated). Green and sustainable development of a meta-industry could enhance profit increase, through the production and sale of new added-value products, while protecting the environment and acting as an antidote to climate change.

The dairy and cheese-making industry have always been pillars of the Greek economy, with a variety of products being widely accepted by the consumers worldwide. Cheese-making practice, however, leads to the production of cheese whey (CW), after milk coagulation and curd drainage [4]. According to Sultana et al. [5], approximately 160×10^6 t. of CW are, annually, produced worldwide, of which 45×10^6 t. are produced within the EU level. CW characteristics depend on milk type and cheese making procedure, containing (per weight) 93–94% moisture, 5–6% lactose, 0.6–1.0% whey proteins and low quantities of mineral salts [6]. In Greece, goat's and sheep's milk is the most widely used [7] for production of cheeses such as Feta and Graviera, and the total CW production is about 700,000 t. per year, with 250,000 t. being used for whey cheeses production, such as Mizithra, Anthotyros, Manouri, and Xynomizithra [8]. CW is a by-product with a high organic load (COD between 50 and 80 g/L and BOD_5 between 35 and 60 g/L) [4,9]. Various ways of reducing the pollutant load, such as anaerobic digestion [10–13], dark fermentation [14,15], usage as animal feed [16], filtration to separate whey protein concentrates, or directly its dehydration (through mostly lyophilization process) and its subsequent use as food additives or supplements in athletes' diet [17,18], have been proposed. On the other hand, cultivation of microorganisms into lactose-based substrates, to synthesize microbial compounds including biomass (and also mycelial mass of pharmaceutical mushrooms) [19–23], single-cell oil (microbial oil) [19,24–27], organic acids, such as lactate [28,29], ethanol [30,31], microbial biosurfactants [32], etc., have been reported in the literature. Second CW is the deproteinized by-product resulting from the manufacture of whey cheese (Mizithra, Anthotyro, Manouri), which is produced by thermal aggregation of whey proteins (at c. 90 °C), or from the deproteinization through filtration and is described as a yellow-green liquid, rich in lactose and salts [27].

Single cell oils (SCOs), lipids produced by single celled entities (viz. yeasts, molds, algae, and bacteria, which accumulate lipids to more than 20% w/w of total biomass), present a great industrial interest as alternative source of animal and vegetable oil and as starting materials for the synthesis of the so-called "second generation" biodiesel and various types of oleochemicals [3,33]. Oleaginous microorganisms, cultivated on sugars or related substrates, accumulate lipids through the de novo lipid production pathway when

a nutrient source, mainly nitrogen, is exhausted. Through an anabolic biochemical process, by virtue of quasi-inverted β-oxidation reactions, acetyl-CoA generates fatty acids, which are then esterified with glycerol, generating structural and storage lipid compounds [34,35].

The selection of microorganisms that could grow on a by-product like second CW, which contains mostly lactose as microbial carbon source, presupposes their capability to hydrolyze the disaccharide into glucose and galactose and subsequently to catabolize the liberated monosaccharides. Several studies have indicated the utilization of pretreated or non-pretreated CW, potentially blended with other co-substrates, for the cultivation of oleaginous yeasts such as *Candida curvata* D [36], *Apiotrichum curvatum* (*Cryptococcus curvatus*) ATCC 20509 [37,38], various *C. curvatus* stains [24], *C. curvatus* KCTC 27583 [39], *C. laurentii* 11 [25], *Yarrowia lipolytica* B9 [40], *Debaryomyces etchellsii* BM1 [41], *Wickerhamomyces anomalus* [42], *C. curvatus* NRRL Y-1511 [26,27], *C. laurentii* UCD 68-201 [27], and *Cystobasidium oligophagum* JRC1 [43]. Lipids that are produced through CW valorization from the mentioned yeast strains, given their abundance in monounsaturated fatty acids, could be used as non-conventional fatty materials amenable to be used in the synthesis of second generation biodiesel [33,34]. On the other hand, and given that in the last years there has been a constant search for the identification of "new" and "non-conventional" proteins amenable to be used as feed or food supplements [34,35], (lipid-rich) yeast cells produced through CW valorization could be also used for this purpose, since most of the mentioned yeast species (i.e., *D. hansenii*, *Y. lipolytica*, *C. curvatus*, etc.) as also many other oleaginous fungi have not been reported to produce toxic compounds or substances with "anti-nutritional" properties (in contrast, these microorganisms produce lipids that contain valuable cellular fatty acids) [34,35]. Some of these such as *Y. lipolytica* have already been classified as GRAS-type microorganisms [34]; therefore, another viable option would be the valorization of CW-based substrates by yeast fermentations in order to produce (lipid-rich) yeast biomass, amenable to be used as food or feed supplement.

The aim of this study was the valorization of the second CW, through bioprocesses. The experiments were performed, initially batch-wise in a defined substrate with CW-derived lactose as sole carbon source and nitrogen limitation, to evaluate the capability of lactose consumption and microbial oil production. Thereafter, trials were performed batch-wise on CW, and thereafter, the most promising strain (*C. curvatus* ATCC 20509) was cultured in fed-batch mode with CW used as substrate in which intermittent additions of concentrated lactose occurred. Physiological considerations were critically discussed.

2. Materials and Methods

2.1. Microorganisms

The used microorganisms were as follows: *C. curvatus* ATCC 20509, *C. curvatus* NRRL ϒ-1511, *C.* (*Naganishia*) *uzbekistanensis* NRRL Y-44, *Trichosporon ovoides* ACA-DC 5052, *Debaryomyces hansenii* ACA-DC 5079, *Papiliotrema laurentii* NRRL Y-2536, and *P. laurentii* NRRL YB-3594. The strains with the code ACA-DC were kindly provided by the Culture Collection of the Laboratory of Dairy Research, Department of Food Science and Human Nutrition, Agricultural University of Athens (Athens, Greece). The strain with the code ATCC was purchased by the American Type Culture Collection (Manassas, VA, USA). Finally, the strains with the codes NRRL Y- and NRRL YB- were provided by the NRRL Culture Collection (Peoria, Illinois, USA). All strains were stored in glycerol solution (50% *v/v*) in freezing (T = −18 °C) and were maintained on YPDA medium (yeast extract at 10 g/L, peptone at 20 g/L, glucose at 20 g/L, agar at 20 g/L) at T = 4 °C. All microorganisms were regenerated in YPDA and were incubated for 24–48 h at T = 30 °C once per month to maintain their viability.

2.2. Media

The initial screening of yeast strains on a semi-defined medium included cultures on a nitrogen-limited medium (inducing the de novo lipid production process; see: [34,35]), where the initial concentration of lactose (carbon substrate; S) was adjusted to c. 55 g/L,

close to the concentration of lactose in second CW [27]. The molar ratio C/N was set at c. 56 moles/moles as a suitable ratio for lipid accumulation, according to Carota et al. [27]. Yeast extract (Y.E., ≈10% *w*/*w* nitrogen content) was used as an organic nitrogen source (2.32 g/L), whereas the concentration of inorganic source, ammonium sulfate, was 1.09 g/L (A.S., ≈21% *w*/*w* nitrogen content). The medium salt concentration was (in g/L): $MnSO_4 \times H_2O$ 0.06, $MgSO_4 \times 7H_2O$ 1.5, $ZnSO_4 \times 7H_2O$ 0.02, $CaCl_2 \times 2H_2O$ 0.15, $FeCl_3 \times 6H_2O$ 0.15, KH_2PO_4 7.0, and Na_2HPO_4 2.5 [44]. The medium was transferred into 250-mL Erlenmeyer flasks (50 ± 1 mL working volume), sterilized in autoclave (T = 121 °C, for ≥20 min), and then inoculated with 1.0 mL of 24-h exponential pre-culture (1–3 × 10^7 cells; initial concentration of biomass—X_0 = 0.12 ± 0.02 g/L in flasks). All pre-cultures were carried out in 250-mL flasks filled with 50 ± 1 mL working volume of the following medium: 5 g/L D-Lactose, 5 g/L D-Glucose, 10 g/L Y.E., and 10 g/L peptone. The cultures were incubated in an orbital shaker (Lab-Line, Illinois, USA) at incubation temperature T = 30 ± 1 °C and 180 ± 5 rpm agitation rate for efficient oxygen transfer. It was necessary to maintain a pH value at 5.5 ± 0.3 (pH/mV meter HI 8014, Hanna Instruments Hellas, Athens, Greece); therefore, an appropriate volume of NaOH (5M) or HCl (5M) was added periodically into the flasks, under aseptic conditions, when it was needed.

The microorganism with the higher potential of production of dry biomass and lipids (viz. *C. curvatus* ATCC 20509) was, furthermore, batch-cultured on lactose-based substrates with nitrogen excess and nitrogen limitation in flasks. The nitrogen excess medium contained initial lactose (S_0) *c.* 46 g/L as carbon source and Y.E. (5 g/L) and A.S. (5 g/L) as nitrogen sources, with C/N ratio equal to 14 ± 1 moles/moles. The nitrogen-limited experiments were conducted in four S_0 concentrations, namely ≈58 g/L, ≈118 g/L, ≈107 g/L, and ≈153 g/L, and the nitrogen sources were added in the appropriate quantities (g/L), Y.E. 1.16 and A.S. 0.545; Y.E. 2.32 and A.S. 1.09; Y.E. 0.5 and A.S. 0.5; Y.E. 0.5 and A.S. 0.5, respectively, to achieve the respective C/N ratios ≈124, ≈124, ≈337, and ≈482 moles/moles. The medium salt concentration and incubation conditions were the same, as described previously.

Second cheese whey (CW) was also employed as a carbon source. It was provided by the Laboratory of Dairy Research (Agricultural University of Athens). The cheese-making process included sheep milk, rennet (90% Chymosin, Vlachopoula SA, Athens, Greece), and lyophilized starter culture of lactic acid microorganisms (Laboratorio PRODOR, Mezzano Scotti, Italy), to produce Feta cheese. The main by-product generated by Feta cheese manufacture, namely CW, was thermally processed at T = 90 °C for half an hour, in order for the whey proteins to be flocculated and collected as whey cheese "Mizithra". The final by-product of Mizithra whey cheese manufacture is the partially deproteinized second CW. The pretreatment of second CW involved centrifugation at 15,000× *g*, at T = 2 °C for 15 min (Sorvall LYNX 6000 Superspeed Centrifuge, Thermo Scientific, Waltham, MA, USA) and filtration of supernatant through a Whatman qualitative filter paper "Grade 5" with a pore size of 2.5 μm. The aim of this treatment was the removal of remnant flocculated proteins. Moisture and total solids content were determined according to the oven-drying method. A sample (5 mL) of treated second CW (after centrifugation and subsequent filtration) was dried at T = 105 °C for 24 h. The total solids content (%) was calculated as follows: $\frac{post/drying\ sample\ (g)}{pre/drying\ sample\ (g)} \times 100$ and the moisture content (%) as $\frac{pre/drying\ sample\ (g) - post/drying\ sample\ (g)}{pre-drying\ sample\ (g)} \times 100$. Furthermore, lactose, total nitrogen, free amino nitrogen, protein, and inorganic phosphorus contents were determined, as described below (see Section 2.3). The characteristics of second cheese whey, as far as pH, total solids, lactose, free amino nitrogen (FAN), total proteins, inorganic phosphorus (IP), and total Kjeldahl nitrogen (TKN) concentrations are concerned, after centrifugation and filtration treatments, are summarized in Table 1.

The next phase concerns the cultivation of primarily screened yeasts in pretreated second CW. The centrifugated and filtrated second CW was transferred to Erlenmeyer flasks (50 ± 1 mL working volume) without adding extra mineral salts and was then sterilized in autoclave (T = 121 °C for 30 min). Thereafter, the inoculum of the pre-culture was

added, and the batch-cultures were incubated, as described above, until the consumption of the carbon source. The strain with the highest production of dry biomass and cellular lipids was also cultivated in 250-mL Erlenmeyer flasks, under fed-batch conditions. The centrifugated and filtrated second CW ($S_0 \approx 60$ g/L) was used as a substrate of fed-batch culture, performed in Erlenmeyer flasks (50 ± 1 mL working volume) without adding extra mineral salts. Intermittent feeding was conducted by a condensed cheese whey-derived lactose (purity ~ 94%, containing 3.5% moisture and 2.5% impurities such as salts) solution (330 ± 5 g/L). When the concentration of lactose was reduced to 10–20 g/L, then appropriate volume of the lactose condensate was added (feeding pulse), so that the lactose concentration of the culture would be of c. 40–60 g/L. The inoculum preparation, inoculation, and cultivation conditions were carried out in the same way as in batch cultures.

Table 1. Characteristics of centrifugated and filtrated second cheese whey.

Total Solids (%)	Lactose (g/L)	FAN (mg/L)	Protein (Bradford) (g/L)	IP (g/L)	TKN (g/L)	pH
7.2 ± 1.0	60.9 ± 1.8	63.0 ± 2.5	1.10 ± 0.15	0.68 ± 0.10	1.14 ± 0.13	6.25 ± 0.21

2.3. Analytical Methods

Flasks were periodically removed from orbital shaker, the culture broth was measured volumetrically (this was needed due to moisture loss attributed to the evaporation), the volume was corrected to 50 ± 1 mL and cells were harvested, as precipitate, through centrifugation at 15,000× *g*, at *T* = 2 °C for 15 min (Hettich Universal Centrifuge, Model 320-R, Merck KGaA, Darmstadt, Germany) and washed twice with distilled water. The supernatant was collected for further analyses. Special treatment was required for the case of *P. laurentii* NRRL Y-2536 cultivation, since fermentation broth was particularly viscous, due to the extra-cellular polysaccharides' secretion. The exopolysaccharides (EPS), in form of capsule and/or released polysaccharides in fermentation broth, as well as the cells were both concomitantly precipitated by anhydrous acetone (purity ≥ 99.5%), but only when the carbon source had been totally consumed. In this case, total dry precipitate consisted of both dried cells and polysaccharides. Acetone was recycled by evaporation (Flash Evaporator/Rotavapor R-114, BÜCHI Labortechnik AG, St. Gallen, Switzerland). Residual lactose (S, g/L), total dry weight (TDW) of biomass (X, g/L), and cellular lipids (L, g/L) were determined (the corresponding methods and assays are analyzed below). pH, yield or substrate to biomass coefficient ($Y_{X/S}$, g of total biomass produced per g of substrate consumed), substrate to lipids coefficient ($Y_{L/S}$, g of cellular lipids produced per g of substrate consumed), dry biomass productivity (P_X, total dry biomass concentration produced per hour expressed in g/L/h), lipid productivity (P_L, total lipid concentration produced per hour, expressed in g/L/h), and lipid content in dry biomass ($K_{L/X}$, g of cellular lipids per g of total biomass) were calculated, according to Papanikolaou et al. [44]. As far as the experiments in second CW were concerned, the following extra analyses were occasionally performed; Free Amino Nitrogen (FAN, mg/L), Total Kjeldahl Nitrogen (TKN, g/L), Residual Protein (RP, g/L), and Inorganic Phosphorus (IP, g/L) assays (as described below). In cases of intra-cellular and/or extra-cellular polymeric substances production, total polysaccharides (TPS, g/L) were determined (as described below).

The total dry biomass (X, g/L) was gravimetrically determined after drying the precipitate at *T* = 80 °C until constant weight (usually for 26–30 h). Total polysaccharides (TPS, g/L), (intra-cellular (IPS) and extra-cellular polymeric substances (EPS)), were determined for *P. laurentii* NRRL Y-2536, according to the slightly modified method of Liang et al. [45] and Argyropoulos et al. [46]. In brief, 50 mg of aggregate (dry weight of yeast cells mass and EPS) were mixed with 2 mL of hydrochloric acid (HCl, 2.5 M) and were allowed to boil (*T* = 100 °C) for 30 min. Cellular debris were removed by centrifugation (as described above) and the supernatant was neutralized with 2 mL of sodium hydroxide (NaOH, 2.5 M). The final volume was adjusted to 5 mL, and the total polysaccharides were quantitatively determined and expressed as glucose equivalents, through the assay

of 3,5-dinitrosalicylic acid [46]. Moreover, the determination of individual saccharides of TPS was performed in selected samples through High-Performance Liquid Chromatography. Filtered aliquots of the neutralized supernatant were analyzed by a Waters Alliance 2695 apparatus at a 30.0 cm × 7.8 mm column Aminex HPX-87H (Bio-Rad Laboratories, California, USA), coupled to a differential refractometer (Refractive Index Detector, Waters 2410). The mobile phase used was H_2SO_4 (0.005 M) with a flow rate of 0.5 mL/min (isocratic elution) and a column temperature of T = 60 °C. The samples were diluted to appropriate concentration and filtered through a 0.2 μm membrane filter before injection (injection volume = 20 μL). The sugars were detected by RI at 880 nm and the results were quantified, corresponding to standards. Total cellular lipids (L, g/L) were extracted from TDW, using the blend of solvents chloroform/methanol (C/M) [47] in a proportion 2:1 v/v, respectively, for 72–96 h, as described in Sarantou et al. [48]. The solvents were recycled by evaporation (Flash Evaporator—Rotavapor R114) and the cellular lipids were gravimetrically determined. A sample (c. 30 mg) of the total cellular lipids was dissolved in C/M 2/1 v/v (1 mL) for Thin Layer Chromatography (TLC) analysis, and the rest was converted to the respective fatty acid methyl esters (FAME) in a two-step reaction, as described in Papanikolaou et al. [44]. TLC was conducted as described by Gardeli et al. [49]; aluminum sheets silica-gel 60 (20 cm × 20 cm × 0.25 mm dimensions—Merck, Darmstadt, Germany) was used for the development of neutral lipids. The plate was activated before use by heating for 2 h at T = 80 °C, then the lipid standards (cholesterol, oleic acid, and triolein at a concentration of 30 mg/mL) and samples were manually injected, and the plate was placed in an all-glass chromatography chamber at room temperature, containing a mixture of solvents (*n*-hexane/diethyl ether/glacial acetic acid—70:30:1, $v/v/v$), as mobile phase. The chromatographic separation (c. 45 min) was followed by drying in a fume hood for 30 min. The visualization was conducted through exposure of the developed TLC plate to iodine vapor in an iodine-saturated chamber. Gas chromatography analysis of FAME was carried out in a GC apparatus (Fisons GC 8000 Series), equipped with a capillary CPWAX 52 CB column (dimensions 60 m × 0.32 mm, film thickness 1.20 μm) and a flame ionization detector (FID, Fisons). Helium (He) is used as the carrier gas, with a gas flow of 2 mL/min; the analysis was isothermally run to T = 240 °C, the temperatures of injector and detector were T = 240 °C and T = 250 °C, respectively, and the FAME were identified and quantified by comparing the retention times and areas of their peaks to those observed for their respective standards (Supelco 37 Component FAME Mix, Sigma-Aldrich, Taufkirchen, Germany). The individual FA content was expressed as weight percentage (g/100 g of total FA).

Residual lactose (Lac, g/L) determination was carried out in Shimadzu Type High Performance Liquid Chromatography (HPLC, Waters Alliance 2695) device, equipped with UV and RI (2414 Refractive Index) detectors, as described above. The determination of free amino nitrogen (FAN, mg/L) of α-amino acids or N-terminals of peptides and proteins was conducted by the ninhydrin photometric method [50]. Total Kjeldahl Nitrogen (TKN, g/L) of second cheese whey cultures were assayed according to Kjeldahl method [51] in a Kjeltek^{TM} 8100 Distillation Unit (Foss A/S, Hillerød, Denmark). Residual protein (RP, g/L) of culture broth was rapidly determined according to the colorimetric method suggested by Bradford [52]. Inorganic phosphorus (IP, g/L) was determined according to the modified method of Elnenaey and Soliman [53]; briefly, samples were properly diluted at a final volume of 5 mL, 0.4 mL of perchloric acid (70% v/v), 0.3 mL of freshly prepared L-ascorbic acid solution (10 g/L), and 0.4 mL of supersaturated ammonium molybdate solution (>50 g/L) were successively added, while the samples were vortexed after each addition. The tubes were left for about 10 min in room temperature in order for the color to be developed, and the absorbance was measured at 730 nm. Solution of dipotassium hydrogen phosphate (K_2HPO_4) was used to prepare a standard curve (0.1 to 10 mg/L inorganic phosphorus).

2.4. Data Analysis

Each experimental point of all the kinetics presented in the tables and figures is the mean value of two independent determinations, while the standard error (SE) for most experimental points was ≤17%. Data were plotted using Kaleidagraph 4.0.3.0 (Synergy Software 1988–2006) showing the mean values with the standard error mean.

3. Results

3.1. Trials on Lactose-Based Media

The first phase of experiments concerned the batch-cultivation of seven yeast strains on a semi-defined medium with commercial lactose as a carbon source ($S_0 \approx 56$ g/L, initial C/N ≈ 58 moles/moles) in 250-mL shake flasks. The results are summarized in Table 2. Five strains were capable to catabolize lactose and grow. HPLC analysis of the culture fluid for all microorganisms consuming lactose and for all culture phases of the trials performed demonstrated only one peak concerning the detection performed in the RI detector, that of lactose. The size of this peak, as it was anticipated, decreased as the fermentation proceeded, demonstrating assimilation of lactose by the implicated microorganisms. Nevertheless, not any peak of glucose and/or galactose appeared, suggesting that for all strains tested, lactose was consumed without having been previously hydrolyzed, with the hydrolysis and the subsequent transformations of lactose being carried out by the relevant enzymes (β-galactosidase, β-galactoside permease, β-galactoside transacetylase) at the cellular level, in full accordance with the results reported by Tchakouteu et al. [26], dealing with the growth of *C. curvatus* strains on lactose-based carbon- and nitrogen-limited media.

Table 2. Quantitative data originated from kinetics of seven microbial strains, cultivated on a semi-defined medium, with lactose as carbon source, at initial lactose (S0) concentration adjusted to c. 56 g/L and initial molar ratio C/N ≈ 58 moles/moles. Culture conditions as in Section 2; standard error for most experimental points was ≤17%.

Strain	Time (h)	S_{cons} (g/L)	X (g/L)	L (g/L)	$Y_{X/S}$ (g/g)	$Y_{L/S}$ (g/g)	$K_{L/X}$ (g/g)	P_L (g/L/h)
C. curvatus ATCC 20509	69	56.4 ± 3.1	22.0 ± 1.8	3.7 ± 0.4	0.39	0.07	0.17	0.054
C. curvatus NRRL Y-1511	130	55.9 ± 2.0	15.5 ± 1.5	0.8 ± 0.2	0.28	0.01	0.05	0.006
C. uzbekistanensis NRRL Y-44	115	Tr.	n.g.	-	-	-	-	-
T. ovoides ACA-DC 5052	115	Tr.	n.g.	-	-.	-	-	-
D. hansenii ACA-DC 5079	115	27.8 ± 1.4	5.1 ± 0.8	0.6 ± 0.2	0.19	0.02	0.12	0.005
P. laurentii NRRL Y-2536	122	59.0 ± 2.0	16.5 ± 1.4 *	2.0 ± 0.3	0.30	0.04	0.12	0.017
P. laurentii NRRL YB-3594	115	54.7 ± 2.3	12.9 ± 1.8	1.2 ± 0.2	0.24	0.02	0.09	0.010

Tr. < 0.5 g/L; n.g.: non-growth conditions; *: Dry weight of both cells and EPS.

TDW of *C. curvatus* ATCC 20509 was =22.0 g/L, with 3.7 g/L cellular lipids produced (17% of TDW), 0.32 g/L/h biomass productivity, and 0.054 g/L/h lipid productivity in a 69 h-long batch fermentation. *C. curvatus* NRRL Υ-1511 produced 15.5 g/L of TDW (with 5% *w*/*w* of lipid in TDW) at t = 130 h. *P. laurenti* Y-2536 produced higher quantities of dry biomass (X = 16.5 g/L) and lipids (L = 2 g/L) than *P. laurenti* YB-3594 (12.9 and 1.2 g/L, respectively), while both strains consumed significant lactose quantities. For *P. laurenti* Y-2536, the dry weight determined contained both produced EPS and the yeast dry cells. *D. hansenii* ACA-DC 5079 consumed half of the available lactose quantity and produced 5.1 g/L of TDW (12% lipid in TDW) in a 115 h-long batch fermentation. The two

microorganisms *C. uzbekistanensis* NRRL Y-44 and *T. ovoides* ACA-DC 5052 were incapable of assimilating lactose and growing under these culture conditions.

Despite considerations in the literature indicating that an initial C/N molar ratio adjusted to c. 60 moles/moles (as in the screening study presented in Table 2) would be sufficiently high to induce noticeable production of lipids inside the yeast cells [54,55], this seemed not to be the case for the microorganisms used in the present investigation. The microorganism *C. curvatus* ATCC 20509, due to higher production of biomass and lipids and the relatively higher lipid content in dry cell basis as compared to the other strains (see Table 2), was chosen to be batch-cultured on lactose-based substrates in 250-mL Erlenmeyer flasks, in various initial C/N molar ratio media, ranging from nitrogen-excess to significant nitrogen-limited conditions. Therefore, the C/N ratio in nitrogen excess medium was set to c. 14 moles/moles (employment of 46.3 g/L lactose, 5 g/L Y.E., 5 g/L A.S.). Nitrogen-limited experiments were conducted in four different initial lactose concentrations ($S_0 \approx$ 58 g/L, 118 g/L, 107, and 153 g/L), while the initial C/N ratios for the respective S_0 concentrations were ≈124, ≈124, ≈337, and ≈482 moles/moles (see also Section 2). Therefore, in the second set of experiments, the impact of both the initial C/N molar ratio and the S_0 concentration of lactose were studied. The obtained results are summarized in Table 3. The cultivation of *C. curvatus* ATCC 20509 in nitrogen excess medium resulted in high biomass production (X = 20.0 g/L) with only 8% lipid content in TDW; that is an expected result since, as indicated in the previous paragraphs, the de novo lipid production process is only enhanced under nitrogen-limited conditions [34,54,55]. Cultivation in C/N ≈ 124 moles/moles led to X = 22.4 g/L and L = 7.3 g/L ($K_{L/X}$ = 0.33 g/g) in the case of $S_0 \approx$ 58 g/L, while for the significantly high S_0 = 118 g/L, TDW production was =44.0 g/L, with a 36% lipid content (the respective L value was =15.8 g/L). Higher nitrogen limitation, viz. the initial imposed C/N ratio ≈ 337 moles/moles, led to production of 10.2 g/L of TDW containing 4.20 g/L lipid production ($K_{L/X}$ = 0.41 g/g) and a volumetric lipid productivity =0.038 g/L/h, whilst the microorganism consumed only 36 g/L lactose, in a 111 h-long batch culture. Further incubation did not improve lactose assimilation from the growth medium (data not presented). At the highest initial C/N ratio imposed (≈482 moles/moles), a TDW quantity =14.9 g/L containing 58.4% *w/w* lipid content (8.7 g/L total cellular lipids) and 0.054 g/L/h lipid productivity, although lactose consumption, as in the previous trial, was somehow limited (c. 33 g/L within 160 h after inoculation, without tendency of further sugar consumption after more prolongated incubation). For the latter case, also of interest for further discussion is the elevated conversion yield of total lipid produced per unit of sugar consumed ($Y_{L/S}$) that was =0.26 g/g (see Table 3), which is one of the highest values ever reported in the international literature concerning the de novo lipid accumulation process by wild-type microbial strains [33,34,54–57]. From the second set of results, it may be indicated that *C. curvatus* ATCC 20509 was indeed a robust microbial candidate as regards its potential to grow on high-lactose concentration media (significant TDW production and lactose consumption occurred in media with S_0 ranging between 118 and 153 g/L) and the more nitrogen limitation was imposed, the more lipid in TDW (%, *w*/*w*) values increased. On the other hand, lactose consumption was decreased at the trials with excessively high initial C/N molar ratios imposed, suggesting that a "compromise" should be found to obtain simultaneously high lactose uptake, significant TDW production, and remarkable accumulation of lipid inside the cells. Under the present culture conditions, this "compromise" seemed to be the case of the trials with initial molar ratio C/N ≈ 124 moles/moles and high S_0 concentrations imposed. The kinetics in one of the mentioned cases ($S_0 \approx$ 118 g/L, C/N ≈ 124 moles/moles) is shown in Figure 1.

Table 3. Quantitative data originated from kinetics of C. curvatus ATCC 20509, cultivated on a semi-defined medium, with lactose as a carbon source, and various initial lactose (S0) concentrations and C/N molar ratio media. Culture conditions as in Section 2; standard error for most experimental points was ≤17%.

C/N Moles/Moles	S_0 (g/L)	Time (h)	S_{cons} (g/L)	X (g/L)	L (g/L)	$Y_{X/S}$ (g/g)	$Y_{L/S}$ (g/g)	$K_{L/X}$ (g/g)	P_L (g/L/h)
14	46.3	139	46.3 ± 2.0	20.0 ± 1.9	1.5 ± 0.2	0.43	0.03	0.08	0.011
124	58.3	96	58.0 ± 2.7	22.4 ± 2.2	7.3 ± 1.2	0.37	0.13	0.33	0.080
124	118.4	144	118.0 ± 3.6	44.0 ± 2.9	15.8 ± 1.6	0.38	0.13	0.36	0.110
337	107.0	111	36.0 ± 1.8	10.2 ± 1.8	4.2 ± 0.9	0.28	0.12	0.41	0.038
482	152.8	160	33.0 ± 2.1	14.9 ± 2.1	8.7 ± 1.6	0.45	0.26	0.58	0.054

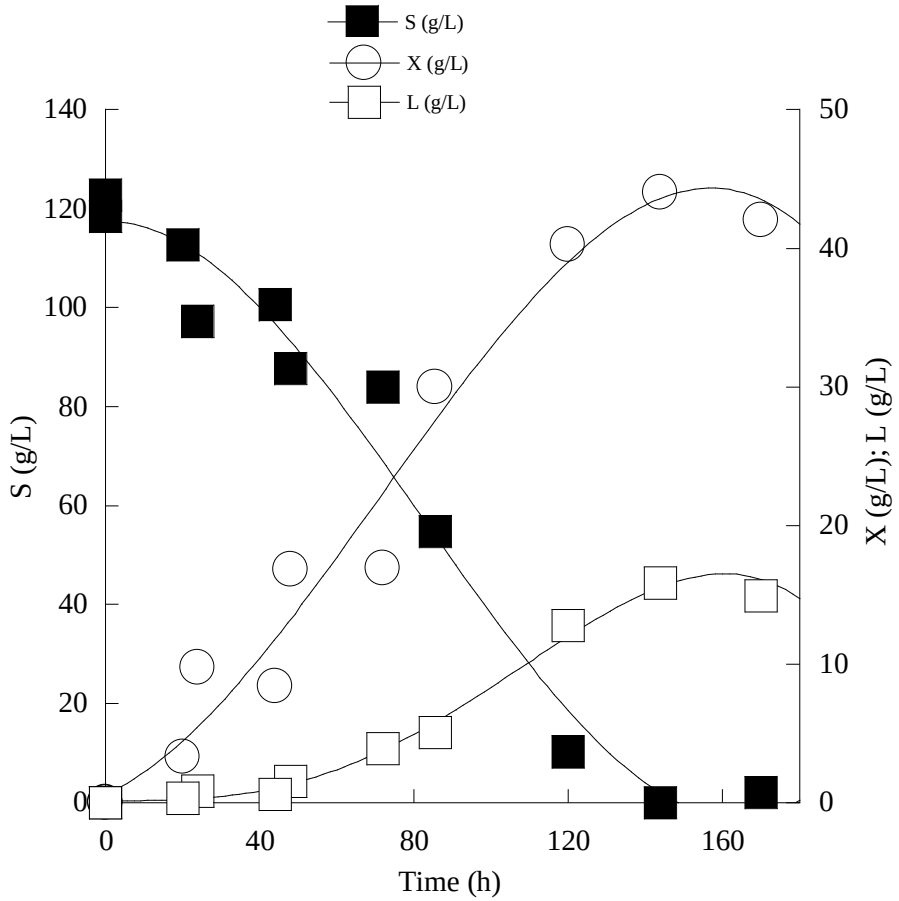


Figure 1. Kinetics of *Cryptococcus curvatus* ATCC 20509 batch cultivation in semi-defined medium, with lactose as carbon source ($S_0 \approx 118$ g/L) and an initial C/N ratio ≈ 124 moles/moles.

3.2. Trials in Treated Second Cheese Whey

The five microorganisms that presented biomass production on lactose-based media, namely *C. curvatus* ATCC 20509, *C. curvatus* NRRL Y-1511, *D. hansenii* ACA-DC 5079, *P. laurentii* NRRL Y-2536, and *P. laurentii* NRRL YB-3594 were batch-cultivated in shake flasks in treated second CW, and the results are presented in Table 4. *C. curvatus* ATCC 20509 fully assimilated lactose, partially consumed FAN (31.6 mg/L), and protein (0.29 g/L), producing 22.4 g/L TDW and 3.4 g/L intracellular lipids (14.7% *w*/*w* of lipids in TDW). *C. curvatus* NRRL Y-1511 also assimilated lactose, partially assimilated FAN (36 mg/L), and protein (0.47 g/L), producing 20.6 g/L TDW and 3.2 g/L intracellular lipids ($K_{L/X}$ = 0.16 g/g). *P. laurentii* NRRL Y-2536 fully consumed lactose, whereas free amino nitrogen (32.1 mg/L) and protein (0.13 g/L) were partially consumed, producing

22.0 g/L TDW and 1.2 g/L intracellular lipids (6% *w*/*w* in TDW). As in the previous set of experiments (screening on lactose-based media) this strain also produced significant quantities of EPS, therefore, dry weight determination included both the produced EPS and the yeast dry cells. Due to the presence of polysaccharides, the texture of biomass was gummy, and the fermentation broth was thick with high viscosity. It was only possible to follow the kinetics of lactose, and one final measurement of biomass was performed after complete lactose consumption (at t = 119 h after inoculation). For *P. laurentii* NRRL YB-3594 culture, lactose was consumed (55.7 g/L), whereas free amino nitrogen (33.3 mg/L) and protein (0.09 g/L) were partially consumed, producing 14.7 g/L dry biomass and 1.0 g/L intracellular lipids (6.9% w/w in TDW) in 101 h of batch cultivation. Finally, concerning the cultivation of strain *D. hansenii* ACA-DC 5079, it consumed only an amount of the available lactose (c. 31% *w*/*w*, viz. 18.1 g/L), producing 8.3 g/L dry biomass with 15% of intracellular lipid content (1.2 g/L) in a 170 h-long batch culture. From all the mentioned analysis, although all tested strains presented interesting (or even noticeable) production of TDW (in most cases the conversion yield $Y_{X/S}$ on consumed lactose was $\geq$0.35 g/g; see Table 4), the accumulation of lipid inside the cells was not dominant ($K_{L/X}$ ranging between 0.07 and 0.16 g/g). Even the high-performing lipid-producing strain *C. curvatus* ATCC 20509, despite complete lactose consumption, produced noticeable TDW quantities containing mediocre lipid amounts (*c.* 15% *w*/*w* of lipid in TDW), suggesting that the initial molar ratio C/N of the employed second CW was inappropriate for lipid accumulation of the implicated strains. Finally, by taking into consideration the polluting load of the residue (viz. the initial concentrations of lactose and proteins; see Table 1), it can be deduced that with the exception of *D. hansenii* ACA-DC 5079, all other employed strains significantly reduced the concentrations of the polluting compounds of CW (lactose and protein; see Table 4), constituting very appropriate microbial candidates amenable to be used for the depollution/detoxification of this residue.

Table 4. Quantitative data originated by kinetics of five microorganisms, cultivated in the complex substrate, treated second cheese whey, with initial lactose (S0) c. 60 g/L. Culture conditions as in Section 2; standard error for most experimental points was $\leq$17%.

Strain	Time (h)	S_{cons} (g/L)	X (g/L)	L (g/L)	$Y_{X/S}$ (g/g)	$Y_{L/S}$ (g/g)	$K_{L/X}$ (g/g)	P_L (g/L/h)	FAN_{cons} (mg/L)	$Protein_{cons}$ (g/L)
C. curvatus ATCC 20509	74	59.0 ± 0.8	22.4 ± 1.2	3.4 ± 0.2	0.38	0.06	0.15	0.045	31.6 ± 3.3	0.29 ± 0.08
C. curvatus NRRL Y-1511	218	59.0 ± 2.0	20.6 ± 1.1	3.2 ± 0.1	0.35	0.05	0.16	0.015	36.0 ± 2.8	0.47 ± 0.10
D. hansenii ACA-DC 5079	170	18.1 ± 1.9	8.3 ± 0.6	1.2 ± 0.2	0.40	0.02	0.15	0.007	41.1 ± 4.5	0.56 ± 0.08
P. laurentii NRRL Y-2536	119	58.0 ± 2.2	22.0 ± 1.5 *	1.2 ± 0.3	0.38	0.02	0.06	0.010	32.1 ± 4.0	0.13 ± 0.05
P. laurentii NRRL YB-3594	101	55.7 ± 2.3	14.7 ± 1.4	1.0 ± 0.2	0.26	0.02	0.07	0.010	33.3 ± 2.9	0.09 ± 0.02

*: Dry weight of both cells and EPS.

3.3. Polysaccharides by *P. laurentii* NRRL Y-2536

As mentioned above, in both the trials on the semi-defined lactose-based medium and the second CW, *P. laurentii* NRRL Y-2536 presented, even since the relatively early growth stages, the production of a "sticky" and "gummy" aggregate, that contained the yeast biomass produced and an extra-cellular "capsule", presumably composed of polysaccharides. It was indeed difficult to separate the biomass, and the whole (viz. biomass and polysaccharide) was isolated after decantation performed by anhydrous acetone (Figure 2). It was also difficult to perform kinetics, and only lactose evolution was followed daily. When lactose was consumed, the "sticky" aggregate was recovered, dry weight determi-

nation (comprising both the microbial mass and the exopolysaccharides) was performed, and the whole aggregate was subjected to hydrolysis and quantification of polysaccharides on glucose equivalents. Indeed, in both trials (semi-defined lactose-based medium and second CW), total polysaccharides (exoplolysaccharides and potentially intra-cellular polysaccharides) were quantified resulting in a quantity of 40–45% *w*/*w* on dry aggregate basis. Analysis of individual sugars, comprising these polysaccharides, revealed that the majority of these polysaccharides was composed of glucose (75–80% *w*/*w* of total polysaccharides) followed by galactose/mannose mixture (25–20% *w*/*w*), providing evidence that the extra-cellular (and potentially intra-cellular to some extent) polysaccharides were mainly composed of glycans and to lesser extent mannans and galactans. Further analysis and study on this point is currently performed by our research team.

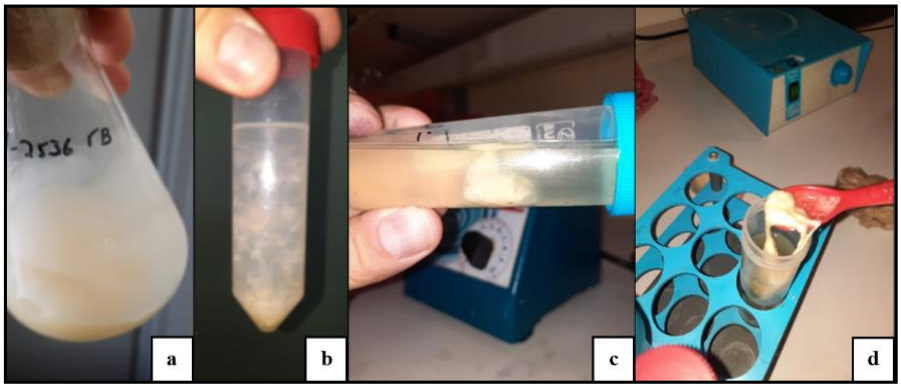
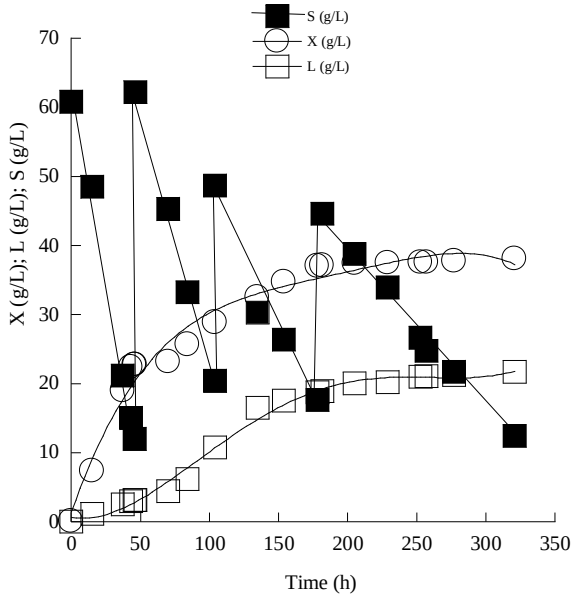


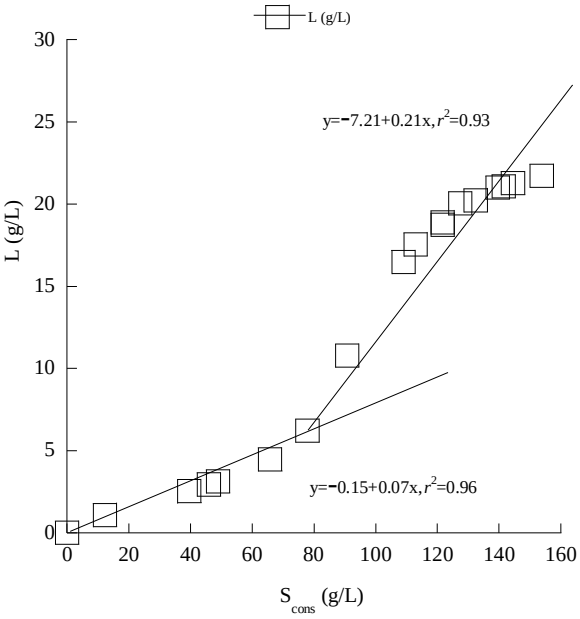
Figure 2. The viscous culture broth of *Papiliotrema laurentii* NRRL Y-2536 cultivation (**a**), after the addition of acetone (**b**), forming a blur (**c**), which precipitates (**d**), resulting in a thick, elastic, gummy precipitate.

3.4. Enhanced Lipid Production through Cultivation of C. curvatus ATCC 20509 in Fed-Batch Conditions

C. curvatus ATCC 20509 was cultivated in treated second CW ($S_0 \approx 60$ g/L) without adding salts or other ingredients into the medium. Thereafter, fed-batch cultivation performed by pulsed feeding with condensed solution of CW derived lactose was carried out. Intermittent lactose feeding was carried out when the remaining, into the medium, lactose was found withing the range of 10–20 g/L. As in all previous cases, trials were conducted in 250-mL Erlenmeyer flasks. The kinetics of the experiment is presented in Figure 3a, and 321 h after inoculation, significant TDW production (X = 38.1 g/L) containing 57% *w*/*w* of lipid in TDW (therefore L concentration was =21.7 g/L) was recorded. A total lactose consumption of 153.9 g/L was observed, whereas the consumed quantities of IP, FAN, protein and TKN of the treated CW were 0.21 g/L, 43.8 mg/L, 0.70 g/L, and 0.41 g/L, respectively. The fed-batch trial can be divided into two well-separated phases, namely the phase in which lactose was mainly consumed in order to produce lipid-free cellular material (in this phase the conversion yield of lipid produced per unit of lactose consumed was c. 0.07 g/g), and the main lipid-producing phase, in which lactose was mainly channeled towards the synthesis of SCO (in this phase, the conversion yield of lipid produced per unit of lactose consumed was c. 0.21 g/g) (Figure 3b). Evidently the second phase of the culture ($Y_{L/S}$ = 0.21 g/g) coincided with the late growth phases, where assimilable nitrogen had already been depleted and lactose was mainly converted to microbial lipid. Microscopic observations throughout the fed-batch culture, demonstrated the evolution of the yeast cells, which as the culture time progressively passed, the size of lipid droplets significantly increased (Figure 4).



(**a**)



(**b**)

Figure 3. (**a**) Kinetics of *C. curvatus* ATCC 20509 fed-batch cultivation in the complex substrate, centrifugated, and filtrated second cheese whey, with initial lactose concentration $S_0 \approx 60$ g/L, presenting the pulsed feeding, dry biomass, and lipid production. (**b**) Fed-batch trial divided into two well-separated phases; the phase in which lactose is mainly consumed in order to produce lipid-free biomass (in this phase the conversion yield of lipid produced per unit of lactose consumed was c. 0.07 g/g), and the main lipid-producing phase, in which lactose was mainly channeled towards the synthesis of SCO (in this phase, the conversion yield of lipid produced per unit of lactose consumed was c. 0.21 g/g).

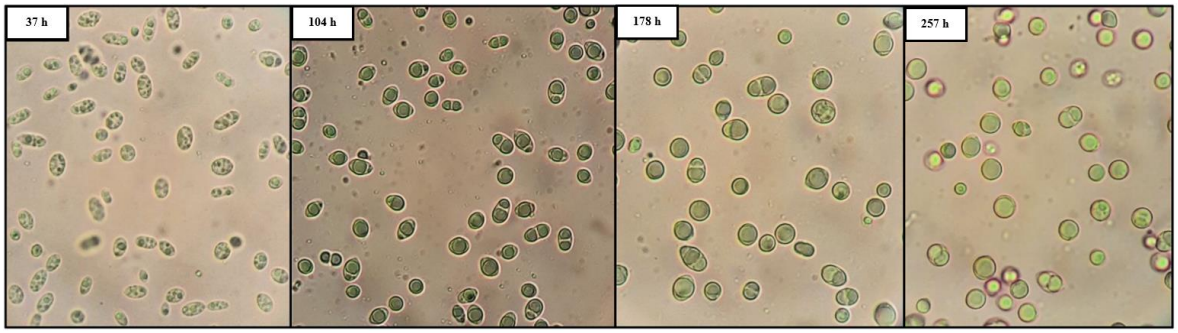


Figure 4. Representation of lipid accumulation progress of *Cryptococcus curvatus* ATCC 20509 fed-batch cultivation in treated second CW supplemented with added CW-derived lactose.

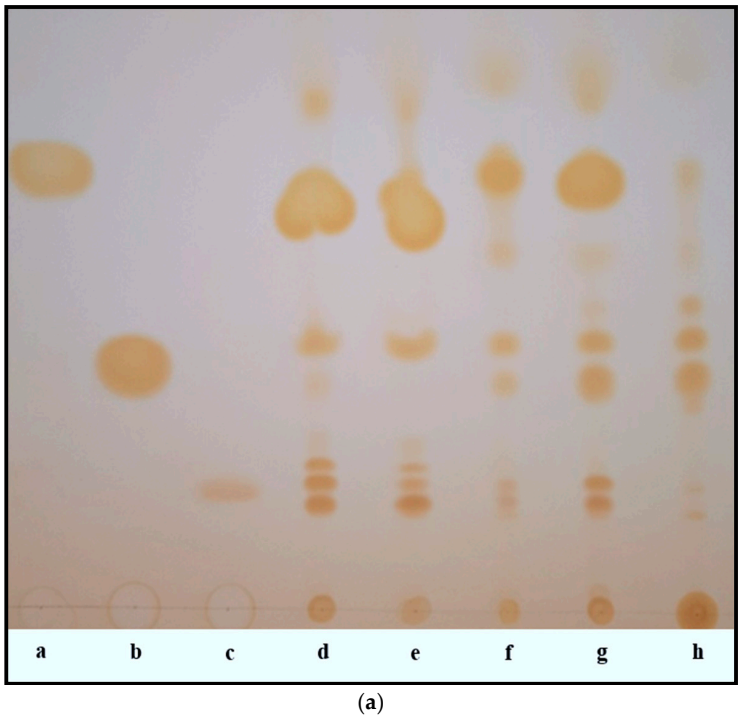
3.5. Yeast Lipid Analysis

All screened strains were analyzed through GC analysis, as regards the fatty acid (FA) composition of the total recovered lipids ("Folch" viz. C/M 2/1 extract) at the stationary growth phase of the trials (FA composition analysis performed at the end of fermentation when lactose was totally assimilated), during cultivation on either lactose-based media or on second CW (the obtained results during growth on second CW are depicted in Table 5a). SCOs of *C. curvatus* ATCC 20509 are mainly composed of unsaturated fatty acids (UFA) (59.9% *w*/*w* of total lipids) and were particularly rich in the $^{\Delta 9}$C18:1 FA (52.8% *w*/*w* of total lipids), while the saturated FAs present a value of 40.1%, being mostly composed of C16:0 (29.3% *w*/*w* of total lipids). FA content of *C. curvatus* NRRL Y-1511 was composed of UFAs (58% *w*/*w*) and saturated FAs (SFA) (42% *w*/*w*). In contrast to ATCC 20509, NRRL Y-1511 lipids included a slightly higher value of poly-unsaturated FAs (PUFA; 9.3% vs. 5.7% *w*/*w*). A difference was observed in the FA composition between the 2 strains of *P. laurentii*. Specifically, FAs of *P. laurentii* NRRL YB-3594 were more unsaturated (65.5% *w*/*w* of total lipids) than them of *P. laurentii* NRRL Y-2536 (53.5% *w*/*w*). This difference is due to the high content of $^{\Delta 9}$C18:1 (56.4% vs. 46.1%) and low content of C16:0 (19.1% vs. 28.2%) FAs. In addition, *P. laurentii* NRRL YB-3594 lipids included higher values of PUFAs (8.3% vs. 4.3% *w*/*w*) than those of *P. laurentii* NRRL Y-2536. As far as the FA content of *D. hansenii* ACA-DC 5079 was concerned, $^{\Delta 9}$C18:1 was the predominant FA (44.8% *w*/*w* of total lipids), followed by C16:0 (26.3% *w*/*w*). The intracellular lipids of this strain were, also, composed of $^{\Delta 9}$C16:1 (12% *w*/*w*), the PUFA content was low (1.9% *w*/*w* of total lipids), but the mono-unsaturated fatty acids (MUFA) content was high (60.4% *w*/*w*). Moreover, the FA analysis concerning the fed-batch culture of *C. curvatus* ATCC 20509, is presented in Table 5b. As the cultivation progressed, intra-cellular lipids of *C. curvatus* ATCC 20509 were principally composed of unsaturated fatty acids (60.1% at 321 h vs. 52.4% at 70 h), rich in $^{\Delta 9}$C18:1 (53.7% of total lipids), while SFAs presented some reduction (47.6% *w*/*w* at 70 h vs. 39.9% *w*/*w* at 321 h), because of C16:0 content decrease (24.3% *w*/*w* at 321 h vs. 36.5% *w*/*w* at 70 h). C18:0 content was slightly increased (14.3% *w*/*w* at 321 h vs. 10.4% *w*/*w* at 70 h). Small quantities of C14:0 (0.5% *w*/*w* of total lipids), 17:0 (0.7% *w*/*w* of total lipids) and C22:0 (0.1% *w*/*w* of total lipids) were observed (not presented). As previously, therefore, FA composition analysis demonstrated predominance of oleic and palmitic acid, constituting thus the cellular lipids of this strain as perfect materials for synthesis of "second generation" biodiesel, in accordance with the literature reports [33]. Total cellular lipids ("Folch" viz. C/M 2/1 extract) were analyzed through TLC analysis (Figure 5a). The strains *C. curvatus* ATCC 20509, *C. curvatus* NRRL Y-1511, *P. laurentii* NRRL Y-2536, and *P. laurentii* NRRL YB-3594 accumulated lipids, mainly in the form of triacylglycerols, although the quantities of storage lipids were not high (in all cases $K_{L/X}$ values were $\leq$0.16 g/g; see Table 4). Other lipid compounds non-identified (i.e., monoacylglycerols, diacylglycerols, phospholipids, ergosterol, and steryl-esters, the latter one presumably being revealed at the top of the TLC plate; see: [49]) were also detected in non-negligible quantities. Another interesting result was associated with the strain

D. hansenii ACA-DC 5079, in which the cellular lipids were principally observed in the form of free FAs (and potentially other not identified in the currently presented work forms, such as monoacylglycerols and diacylglycerols) and to lesser extent as triacylglycerols. TLC analysis of the lipids, derived by fed-batch cultivation of *C. curvatus* ATCC 20509, also demonstrated that, even from the early growth steps, the microorganism mostly accumulates its total cellular lipids (C/M 2/1 extract) in the form of neutral compounds and, principally, triacylglycerols (Figure 5b).

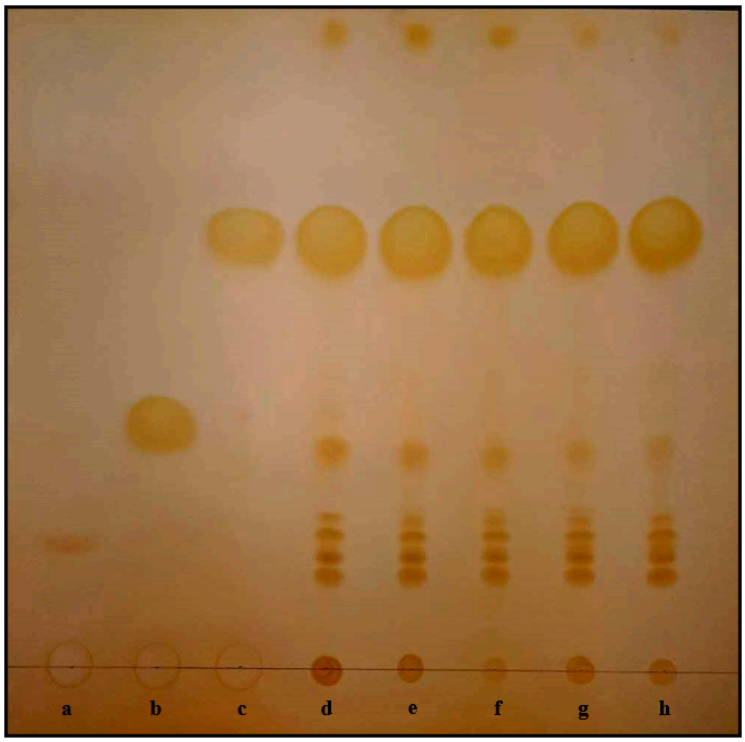
Table 5. (a) Fatty acid composition of the cellular lipids produced by yeast strains cultivated on second CW in shake-flask experiments at the stationary phase of microbial growth. (b) Fatty acid composition of the cellular lipids produced by *C. curvatus* ATCC 20509 at two different time-points, during fed-batch cultivation on second CW. The individual FA content was expressed as weight percentage (g/100 g of total FA).

Strain	g/100 g of Total FA										
	C14:0	**$^{\Delta 9}$C14:1**	**C16:0**	**$^{\Delta 9}$C16:1**	**C18:0**	**$^{\Delta 9}$C18:1**	**$^{\Delta 9,12}$C18:2**	**SFA**	**UFA**	**MUFA**	**PUFA**
(**a**)											
C. curvatus ATCC 20509	0.5	0.2	29.3	1.2	10.3	52.8	5.7	40.1	59.9	54.2	5.7
C. curvatus NRRL Y-1511	0.8	1.2	26.3	0.2	14.9	47.3	9.3	42.0	58.0	48.7	9.3
P. laurentii NRRL Y-2536	3.6	2.5	28.2	0.6	14.7	46.1	4.3	46.5	53.5	49.2	4.3
P. laurentii NRRL YB-3594	0.9	0.5	19.1	0.3	14.4	56.4	8.3	34.5	65.5	57.2	8.3
D. hansenii ACA-DC 5079	5.1	3.7	26.3	12.0	6.2	44.8	1.9	37.7	62.3	60.4	1.9
(**b**)											
C. curvatus ATCC 20509	0.7	-	36.5	1.0	10.4	46.6	4.8	47.6	52.4	47.6	4.8
	0.5	-	24.3	0.6	14.3	53.7	5.7	39.9	60.1	54.4	5.7



(**a**)

Figure 5. *Cont.*



(**b**)

Figure 5. (**a**) Separation of neutral lipids fractions, extracted by the five strains dry biomass, cultivated in second CW through thin layer chromatography. a: triacylglycerol standard; b: free fatty acids standard; c: cholesterol standard; d: *C. curvatus* ATCC 20509/74 h; e: *C. curvatus* NRRL Y-1511/218 h; f: *P. laurentii* NRRL Y-2536/119 h; g: *P. laurentii* NRRL Y-3594/101 h; h: *D. hansenii* ACA-DC 5079/170 h of cultivation. (**b**) Separation of neutral lipids fractions, extracted at different time spots during the fed-batch cultivation of *C. curvatus* ATCC 20509, through thin layer chromatography. a: cholesterol standard; b: free fatty acids standard; c: triacylglycerol standard; d: 70 h; e: 154 h; f: 178 h; g: 257 h; h: 312 h of cultivation.

4. Discussion

Meta-industry, as a term introduced for the first time in this paper, concerns the sustainable waste valorization amenable for the simultaneous pollutant load reduction and the subsequent added-value metabolites production. Dairy and cheese-making industries have always been pillars of the economy, in both international and national (Greek) level. However, cheese-making practice leads to the production of enormous cheese whey quantities, a by-product with high organic load. In the present study, sustainable and eco-friendly conversions of second CW, a pollutant wastewater with destructive effects when released into the environment, but with novel products when properly utilized [4,23,32], were performed and important microbial metabolites (yeast biomass, lipids, and polysaccharides) were produced. In our research, the capability of seven yeast strains to catabolize lactose and accumulate lipids was evaluated. Out of the seven strains, five of them were capable to assimilate lactose without previously hydrolyzing it at the extra-cellular level (in accordance with Tchakouteu et al. [26]) and grow on this medium. Subsequently, these microorganisms were cultivated in pretreated second CW, without any addition of extra elements. CW was primarily used for whey cheese "Mizithra" manufacture, and the second CW, which was left over after this process, was treated through centrifugation and filtration, to remove protein substances.

From the obtained results, it was revealed that *C. curvatus* ATCC 20509 demonstrated the highest potential concerning SCO production, and, for this reason, it was further studied in trials with different C/N and initial lactose concentrations, but also in fed-batch trials with second CW and pulsed lactose additions into the medium. In the current culture condi-

tions, it has been indicated that the initial C/N ratio ≈56 moles/moles, although, according to the literature being considered as quite high and amenable to promote considerable lipid accumulation [27,33,54,55,57], it was revealed as inappropriate to induce noticeable storage of lipid inside the yeast structures. On the other hand, C/N ratios >100 moles/moles seemed capable to enhance intra-cellular lipid storage, especially for *C. curvatus* ATCC 20509. Specifically, increased biomass (=44 g/L), containing 36% *w*/*w* intracellular lipids, was recorded for this strain at initial C/N ratio adjusted to c. 124 moles/moles and initial lactose concentration ≈118 g/L (Table 3). Moreover, fed-batch culture, initiated with treated second CW and accomplished with pulsed lactose quantities, resulted in high lipid accumulation, 57% *w*/*w* of lipids content (21.7 g/L) in 38.1 g/L of total dry biomass.

Various treatment techniques have been applied in second CW to reduce protein content and achieve high lipid accumulation. C/N ratio increase (from 25 to 70 mole/mole) in *Apiotrichum curvatum* ATCC 20509 cultures, led to higher lipid content (from 18% to 58% *w*/*w*) [37]. Cultivation of *Candida curvata* D in ultra-filtrated cheese whey permeate led to 13.8 g/L dry biomass with 55% intra-cellular lipids [36]. In their research, Seo et al. [39] used hydrodynamic cavitation (HC), a high-pressure technique, combined with alkaline condition (pH 9-12) as second CW treatment. Cultivation of *Cryptococcus curvatus* KCTC 27583 in this substrate led to 7.2 g/L dry biomass and 4.68 g/L lipid production, with high values of lipid content and productivity (65% of total biomass and 0.195 g/L/h, respectively), indicating that this pretreatment led to accumulation of intra-cellular lipids. *Cryptococcus curvatus* NRRL Y-1511 produce high quantities of dry biomass (38.5 g/L) [26] but can also accumulate lipids (6.8 g/L, 63% of TDW) [27]. In the case of *Cryptococcus laurentii* UCD 68-201, the highest lipid content was observed in batch cultivation, both in flasks (70% lipid content) and bioreactor (69% lipid content) [28]. Cultivation of *Debaryomyces hansenii* strains in second CW substrate, to produce biomass and microbial lipids, according to our knowledge has not been reported yet. On the other hand, *Debaryomyces etchellsii* BM1 produced low quantities of dry biomass (3.9 g/L) and lipids (0.4 g/L), when cultivated in deproteinized cheese whey [41], in contrast to *Yarrowia lipolytica* B9, which produced 7.4 g/L dry biomass and 4.3 g/L lipids (58% of TDW) [40]. As far as the microorganism *Cystobasidium oligophagum* JRC1 was concerned, cultivation on untreated CW led to high biomass production (20.98 g/L) and low lipid content (22% of TDW), but the lipid content increased (44% of TDW) when was cultivated in centrifugated and filtrated SCW, due to low values of nitrogen content [43].

Co-utilization of second CW and other agro-industrial residues and by-products such as wine lees and molasses has also been reported; batch culture of *Cryptococcus curvatus* ATCC 20509 in ultrafiltrated CW permeate, supplemented with wine lees hydrolysate, led to 33.6 g/L dry biomass production (30.6% lipid content), when cultivated in flasks, but fed-batch cultivation in bioreactor led to even higher lipid content (49.6%), according to Kopsahelis et al. [38]. *Cryptococcus laurentii* 11, was cultivated in centrifugated CW used as sole substrate, and produced 4.6 g/L dry biomass and 1.3 g/L lipids. When the substate was supplemented with molasses, the dry biomass production was, interestingly, increased (16.6 g/L), but not the lipid production [25]. Literature results dealing with growth of oleaginous yeasts on CW- and lactose-based media and their comparisons with the best results achieved in this study are recorded in Table 6.

Table 6. Literature review and comparative evaluation regarding to biomass and lipid production and biomass and lipid productivity by oleaginous yeasts, cultivated on pre-treated cheese whey substrate. Comparisons with the present study.

Strain	Culture Conditions	C/N	Substrate	X (g/L)	L (g/L)	K_X^L (g/g)	Biomass Productivity (g/L/h)	Lipids Productivity (g/L/h)	Reference
Apiotrichum curvatum ATCC 20509	Batch b/r, pH = 4.8, t = 27 h	25	Ultrafiltrated CW Permeate C/N adjustment: NH_4Cl	23.20	4.2	0.18	0.86	0.155	[36]
	Batch b/r, pH = 4.8, t = 39 h	40		21.60	5.6	0.36	0.56	0.199	
	Batch b/r, pH = 4.8, t = 93 h	70		19.70	11.4	0.58	0.21	0.123	
	Fed-batch b/r, pH = 4.8, t = 70 h	40		85.00	29.8	0.35	0.43	0.372	
	Continuous b/r, pH = 4.8, D = 0.07 h^{-1}	20		21.00	4.2	0.20	1.40	0.294	
	Continuous b/r, pH = 4.8, D = 0.053 h^{-1}	40		20.00	7.2	0.36	1.10	0.382	
	Partial recycling b/r, pH = 4.8, D = 0.033 h^{-1}	40		91.40	30.2	0.33	3.00	0.995	
Cryptococcus curvatus KCTC 27583	Batch shaken baffled flasks, pH = 5.5, t = 24 h	n.a.	Alkaline Hydrodynamic Cavitation in CW	7.20	4.7	0.65	0.30	0.195	[38]
Cryptococcus curvatus NRRL Y-1511	Batch shaken flasks, pH = 5.5, t = 72 h	55	Ricotta Second CW C/N adjustment: $(NH_4)_2SO_4$	10.77	6.8	0.63	0.15	0.094	[26]
Cryptococcus curvatus NRRL Y-1511	Batch shaken flasks, pH = 5.5, t = 216 h	n.a.	Concentrated and deproteinized CW	38.50	1.4	0.04	0.19	0.006	[25]
Cryptococcus curvatus ATCC 20509	Batch shaken flasks, pH = 6.0, t = 216 h	n.a.	Ultrafiltrated CW Permeate + Wines lees hydrolysate	33.60	6.7	0.31	0.16	0.030	[37]
	Fed-batch b/r, pH = 6, t = 100h	n.a.		66.80	33.1	0.50	0.67	0.490	
Candida curvata D	Batch b/r, pH = 5.4, t = 80 h	n.a.	Ultrafiltrated Cheddar CW Permeate	13.80	7.6	0.55	0.17	0.095	[35]
	Continuous b/r, pH = 5.4, D = 0.02 h^{-1}	n.a.		14.20	7.2	0.51	0.28	0.144	
Cryptococcus curvatus ATCC 20509	Batch shaken flasks, pH = 5.5, t = 74 h	58	Centrifugated and Filtrated Mizithra Second CW	22.40	3.3	0.15	0.30	0.045	Current Study
	Fed-Batch shaken flasks, pH = 5.5, t = 321 h	182	Centrifugated and Filtrated Mizithra Second CW supplemented with condensed CW derived lactose	38.10	21.7	0.57	0.12	0.070	
Cryptococcus curvatus NRRL Y-1511	Batch shaken flasks, pH = 5.5, t = 218 h	58	Centrifugated and Filtrated Mizithra Second CW	20.60	3.2	0.16	0.09	0.015	Current Study

Table 6. *Cont.*

Strain	Culture Conditions	C/N	Substrate	X (g/L)	L (g/L)	K_X^L (g/g)	Biomass Productivity (g/L/h)	Lipids Productivity (g/L/h)	Reference
Cryptococcus laurentii UCD 68-201	Batch shaken flasks, pH = 5.5, t = 72 h	55	Ricotta Second CW C/N adjustment: $(NH_4)_2SO_4$	7.28	5.1	0.70	0.10	0.070	[26]
	Batch Bioreactor pH = 5.5, t = 60 h			14.37	9.9	0.69	0.24	0.165	
Cryptococcus laurentii 11	Batch shaken flasks, pH = 5.5, t = 240 h	n.a.	Centrifuged CW	4.57	1.3	0.28	0.02	0.005	[24]
	Batch shaken flasks, pH = 6.5, t = 360 h	n.a.	Centrifuged CW + molasses	16.58	1.5	0.09	0.05	0.004	
Papiliotrema laurentii NRRL Y-2536	Batch shaken flasks, pH = 5.5, t = 119 h	58	Centrifugated and Filtrated Mizithra Second CW	22.00	1.2	0.06	0.19	0.010	Current Study
Papiliotrema laurentii NRRL YB-3594	Batch Shaken flasks, pH = 5.5, t = 100.5 h	58	Centrifugated and Filtrated Mizithra Second CW	14.70	1.0	0.07	0.15	0.010	
Yarrowia lipolytica B9	Batch shaken flasks, pH = 6.0, t = 120 h	n.a.	Deproteinized Cheese Broth	7.40	4.3	0.58	0.06	0.035	[39]
Cystobasidium oligophagum JRC1	Batch shaken flasks, pH = 6.5, t = 168 h	~60	Untreated CW	20.98	4.6	0.22	0.13	0.030	[42]
		93	Centrifugated and Filtrated Second CW	12.79	5.6	0.44	0.08	0.033	
Debaryomyces etchellsii BM1	Batch shaken flasks, pH = 6.0, t = 120 h	n.a.	Deproteinized CW	3.90	0.4	0.10	0.03	0.003	[40]
Debaryomyces hansenii hansenii ACA-DC 5079	Batch shaken flasks, pH = 5.5, t = 170 h	58	Centrifugated and Filtrated Mizithra Second CW	8.30	1.2	0.15	0.05	0.007	Current Study

b/r: bioreactor; n.a.: not available.

Despite the restricted lipid accumulation process that was observed during growth of yeasts on second CW ($K_{L/X}$ < 16% *w*/*w*; see Table 2), triacylglycerols were the major lipid compound identified for almost all strains tested. This is in agreement with results reported by other oleaginous and non-oleaginous microorganisms (i.e., *Yarrowia lpolytica*, *Cunninghamella echinulata*, *Thamnidium elegans*, etc.), which, irrespective of the imposed culture conditions and the subsequent low or high lipid accumulation observed, produced cellular lipids that were in most cases rich in neutral fractions and mostly triacylglycerols [58–60]. On the other hand, in agreement with the literature [3,33,34,55], the principal FAs found in variable quantities inside the studied yeast lipids were mainly oleate and palmitate and to lesser extent linoleate and stearate (Table 5), being therefore potential candidates for the synthesis of second generation biodiesel, oleochemicals, and biolubricants [33,59]. PUFAs were not detected in high concentrations, since these compounds are the principal storage lipophilic compounds in oleaginous fungi and algae and, in general, they can be produced in significant quantities inside the yeast cells only after appropriate genetic modifications [35]. Finally, a new strain not extensively utilized in the literature, namely *Papiliotrema laurentii* NRRL Y-2536, produced significant quantities of total dry biomass (22.0 g/L) and secreted exopolysaccharides, mainly composed of glucose, galactose, and mannose.

5. Conclusions

Second CW and CW-derived lactose were competitive carbon sources of several wild-type yeast strains. Storage lipid production presupposes low nitrogen content, so the deproteinization of CW through production of whey cheese, such as Mizithra, or whey protein concentrates, is necessary. In this study, centrifugation and filtration treatment of second CW reduced nitrogen content, but not in appropriate values to enhance lipid accumulation. Fed-batch conditions enhanced lipid accumulation due to pulsed whey-derived lactose supply. Cellular lipids contained in variable quantities the fatty acids $^{\Delta 9}$C18:1, C16:0, C18:0, and $^{\Delta 9,12}$C18:2, constituting perfect candidates for the synthesis of "second generation" biodiesel, oleochemical and lubricants. Finally, microbial polysaccharides (extra- or intra-cellular), presenting very high potential for the food and nutraceutical industries, can also be produced in significant concentrations, while in any case, dry biomass of oleaginous yeasts could be utilized as a nutrient-rich animal feed. Overall, CW and CW-lactose constituted very interesting substrates in order for yeast biomass, SCOs, and polysaccharides to be synthesized. Scale-up of the process in laboratory-scale reactors is envisaged in the near future, in order to further study and optimize the mentioned and very interesting bioprocesses.

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