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Agro-Food and Lignocellulosic Urban Wastes as Sugar-Rich Substrates for Multi-Product Oil-Based Biorefineries

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Abstract

The effective use of biowaste resources becomes crucial for the development of bioprocessing alternatives to current oil- and chemical-based value chains. Targeting the development of multi-product biorefinery approaches benefits the viability and profitability of these process schemes. Certain oleaginous microorganisms, such as oleaginous red yeast, can co-produce industrially relevant bio-based products. This work aims to explore the use of industrial and urban waste as cost-effective feedstock for producing microbial oil and carotenoids using *Rhodosporidium toruloides*. The soluble fraction, resulting from homogenization, crushing, and centrifugation of discarded vegetable waste, was used as substrate under a pulse-feeding strategy with a concentrated enzymatic hydrolysate from municipal forestry residue obtained after steam explosion pretreatment (190 °C, 10 min, and 40 mg H_2SO_4/g residue). Additionally, the initial nutrient content was investigated to enhance process productivity values. The promising results of these cultivation strategies yield a final cell concentration of 36.4–55.5 g/L dry cell weight (DCW), with an intracellular lipid content of up to 42–45% (*w*/*w*) and 665–736 µg/g DCW of carotenoids. These results demonstrate the potential for optimizing the use of waste resources to provide effective alternative uses to current biowaste management practices, also contributing to the market of industrially relevant products with lower environmental impacts.

Keywords: single-cell oil; biowastes; oleaginous yeast; carotenoids; advanced biofuels

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Academic Editors: Amanda Laca Pérez, Yolanda Patiño and Daniel Sol

Received: 29 May 2025
Revised: 20 June 2025
Accepted: 24 June 2025
Published: 27 June 2025

Citation: Rodríguez-López, A.; Negro, M.J.; Fernández-Rojo, J.L.; Ballesteros, I.; Moreno, A.D. Agro-Food and Lignocellulosic Urban Wastes as Sugar-Rich Substrates for Multi-Product Oil-Based Biorefineries. *Appl. Sci.* **2025**, *15*, 7240. https://doi.org/10.3390/app15137240

1. Introduction

Multi-product biorefineries play a crucial role in the development and implementation of a sustainable bioeconomy, particularly in the context of significant environmental challenges, including fossil fuel depletion, climate change, and waste management. These facilities target the complete fractionation and valorization of a wide range of biomass resources for producing both bulk and specialty co-products, making the bioprocessing cost-competitive [1]. Biomass residues such as agro-food waste (e.g., discarded vegetables) and lignocellulosic residues (e.g., woody residues, agricultural wastes, and energy crops) are primary raw materials for these multi-product biorefineries [2]. Therefore, the efficient use of these feedstocks is crucial to obtain a wide range of value-added products, including biofuels and other bio-based products with applications in the chemical, pharmaceutical, food/feed, or material sectors. However, the heterogeneity and diverse nature of waste

resources may represent a significant limitation during bioconversion processes [2]. This fact is due to the need to use different methodologies to obtain the sugars contained in these feedstocks (e.g., agro-food residues mainly require crushing and liquid/solid separation steps, while lignocellulose require pretreatment and enzymatic hydrolysis steps), as well as the need of converting a wide range of carbon sources and facing different stress factors (e.g., the presence of inhibitory compounds).

One of the most attractive products that can be obtained from biowastes is microbial oil, also known as Single-Cell Oil (SCO) [3]. The production of microbial oil has grown considerably in recent years due to their potential to replace the vegetable oil used in sectors as diverse as food (nutritional supplements or food additives), energy (biofuels), the pharmaceutical industry (arachidonic acid, docosahexaenoic acid) and the material sector (biopolymers, bioplastics). Yeast, microalgae, or bacteria produce SCO. These microorganisms exhibit faster growth cycles compared to plant crops, can utilize a wide range of carbon sources for lipid accumulation, and can be cultivated under well-controlled conditions without the need to use fertile land, thus extending the possibility of dedicating these areas to other crops. In addition, oleaginous microorganisms can be subjected to genetic engineering to design and develop novel strains, targeting increased process efficiency, providing relevant insights into the conversion pathway, and/or reducing process costs through the simultaneous co-production of value-added compounds [4]. The red yeast *Rhodosporidium toruloides* is an oleaginous microorganism with great biotechnological potential [5–7]. This is due to its capacity to grow on a wide range of carbon sources, its tolerance to different biomass-related inhibitory compounds, and its robustness to other stress factors associated with bio-based processes [8]. In addition to accumulating SCOs, this yeast naturally produces carotenoids, which confer its red colour and are important value-added products with antioxidant properties. Due to their antioxidant and colourant properties, carotenoids may have multiple applications in the food industry (dietary supplements), cosmetics (colourants and photoprotective additives), or the pharmaceutical sector (vitamin precursors, active compounds that modulate the immune system) [9]. Currently, carotenoids are obtained from plants, microbial-based biosynthesis, or chemical synthesis—the synthetic production of carotenoids. However, the growing demand for natural products by global consumers calls for the development of new research with emerging and alternative ways to obtain these products [10]. The Polaris Market Research report valued the global natural beta-carotene market in 2024 at $1.28 billion [11]. These figures are projected to exceed $2.06 billion by 2034. All these advantages make *R. toruloides* an excellent microbial candidate for the conversion of biowastes within a multi-product biorefinery perspective.

Cost-effective carbon sources and media composition are important aspects to consider during SCO production. Nutrient-limiting conditions and high carbon/nitrogen (C/N) ratios are required to trigger lipid production in yeast cells [2]. Sulphur, nitrogen, and phosphorus are the main nutrient-limiting factors utilized to activate lipogenesis [12,13]. However, the stress caused by low nutrient concentration may also result in reduced cell biomass production and/or cell growth inhibition. Nutrient-rich streams are therefore required to boost cell growth at the initial stages of SCO production, thus improving product volumetric productivity and shortening overall process times. In this context, agro-food biowastes and lignocellulosic residues represent attractive raw materials for SCO production as examples of nutrient-rich and nutrient-limiting media, respectively [2,14,15].

In addition to utilizing diverse carbon sources, oleaginous yeast can be cultivated under various processes and strategies to enhance overall conversion yields and/or volumetric productivities [16]. Several schemes have been investigated for SCO production using oleaginous yeasts, including batch operation, fed-batch, and continuous approaches.

Among them, fed-batch and continuous cultivation modes usually exhibit higher conversion yields than the batch operational mode due to improved substrate utilization [17,18]

The use of agro-food industrial waste from the horticulture sector and lignocellulosic residues from urban waste (waste from municipal forestry pruning) as cost-effective carbon sources to produce microbial oil and carotenoid compounds without supplementing external nutrient components is explored. However, scarce literature on this topic is found. The present research assessed the use of both lignocellulosic and non-lignocellulosic residues under different pulse-feeding cultivation strategies to evaluate the intracellular accumulation of lipids and carotenoids in the yeast *R. toruloides*. Cell biomass formation, cell viability, glucose consumption, and intracellular accumulation of lipids and carotenoids were monitored during these processes. Special attention was given to growth rates and final cell biomass concentrations with the aim of evaluating the volumetric productivity during these processes. For that, different initial nutrient concentrations were evaluated by concentrating the agro-food waste stream to avoid external nutrient addition. Overall, the results presented herein will contribute to the development of an effective bioconversion process using biowaste resources, supporting the development of a multi-product biorefinery and providing alternative waste treatment solutions to support sustainability and responsible environmental management.

2. Materials and Methods

2.1. Microorganism and Media Preparation

R. toruloides ATCC 204091 was used to produce lipids and carotenoids. Yeast cells were maintained in 20% glycerol stock at −80 °C. They were activated overnight in 50 mL YPD liquid medium (20 g/L glucose, 10 g/L yeast extract, and 20 g/L peptone) at 180 rpm and 28 °C, using 250-mL baffled conical Erlenmeyer flasks.

Due to their differences in nutrient composition, agro-food waste and lignocellulosic urban residues were used to promote cell growth and trigger intracellular lipid accumulation. The agri-food waste used came from the Spanish horticulture sector, specifically from a mixture of discarded tomatoes, peppers, and cucumbers. These wastes were collected and provided by Fundación Cajamar (Almería, Spain). The soluble fraction obtained after homogenization, crushing, and centrifugation of the waste was used as a culture medium for yeast growth. The discarded vegetables were subjected to crushing using an industrial blender (Dianamix TR/bM 330, SAMMIC, Azkoitia, Spain) and then subjected to centrifugation (3000× *g*, 15 min) using a basket centrifuge RTL2BD (Comfeifa, Barcelona, Spain). The soluble fraction was then collected and filter sterilized using a 0.22 µm Nalgene™ Rapid-Flow™ Sterile Disposable Filter Units (ThermoFisher Scientific, Waltham, MA, USA). With the aim of increasing the initial sugar content, the resulting liquid was also subjected to concentration using a rotavapor (Büchi, Uster, Switzerland) until the initial sugar concentration was doubled. Both non-concentrated and concentrated liquid fractions were analyzed, as described below.

On the other hand, the hydrolysate resulting from subjecting a lignocellulosic urban tree pruning residue to pretreatment and enzymatic hydrolysis was used as a medium for lipid accumulation. This residue was collected and supplied by CEDER-CIEMAT (Soria, Spain). This residue was mainly composed of collected pruning mixtures from elms, plane, and acacia trees. This municipal forestry waste was chopped and milled at 8–10 mm before pretreatment. The residue, whose composition in main components was as follows (% dry weight basis): Cellulose 34.7; hemicellulose 18.7; and Lignin 26.4, was pretreated at CIEMAT-Moncloa (Madrid, Spain) using a 2-L steam explosion reactor designed based on Mansonite technology. The pretreatment conditions (190 °C, 10 min, and 40 mg H_2SO_4/g lignocellulosic biomass) were selected based on previous results, considering solid recovery

after pretreatment, its chemical composition, and the enzymatic hydrolysis yields [19]. After pretreatment, the water-insoluble solids were separated by filtration and subjected to 72 h of enzymatic hydrolysis at 15% (*w*/*w*) solids concentration, an enzyme loading of 20 mg of protein/g glucan (SAE0020, 220 mg protein/mL; Merck, Germany), 50 °C temperature, and pH 5.0. The resulting hydrolysate was collected by filtration, concentrated by vacuum evaporation using a rotavapor until a glucose concentration of about 250 g/L (to prevent dilution during the pulse-feeding cultivation strategy), and then filter-sterilized through a 0.22 μm filter. The concentrated hydrolysate was analyzed prior to its use as described below.

2.2. Pulse-Feeding Cultivation Strategy

R. toruloides ATCC 204091 (purchased through LGC Standards, S.L.U, Barcelona, Spain) was cultivated following a pulse-feeding strategy according to the procedure described by Gallego-García et al. [20]. For that, 0.5-L bioreactors (MiniBio Applikon® Biotechnology, Delft, The Netherlands) with an initial working volume of 250 mL of growth media (i.e., non-concentrated and concentrated liquid fractions obtained from agro-food waste) and operating at 30 °C and pH 6 (pH control with 2 M KOH and 2 M HCl) were used. Antifoam A (Merck, Darmstadt, Germany) was used for foam control with an initial concentration of 0.02% (*v*/*v*). A continuous air supply was used to maintain the aerobic conditions, and the dissolved oxygen level was set at 20% by adjusting the agitation speed (500–1500 rpm). After sugar depletion (i.e., when having a sugar concentration <5 g/L and/or when observing an increase in dissolved oxygen levels), 50 mL of the concentrated enzymatic hydrolysate from the lignocellulosic urban pruning residue was added to trigger lipid accumulation. Before the pulse addition, 50 mL of the corresponding culture was harvested to determine the intracellular lipid content, fatty acid and carotenoid profile, and to evaluate the conversion yields. In order to boost lipid accumulation, two pulses of the enzymatic hydrolysate were added to the corresponding cultures to ensure nutrient-limiting conditions.

Collected cells were then harvested by centrifugation (5000× *g*, 4 °C, and 15 min), washed twice with sterile water, freeze-dried (Telstar LyoQuest, Barcelona, Spain), and stored under dark conditions in a desiccator until further use.

2.3. Analytical Methods

Sugars were analyzed by high-performance liquid chromatography (HPLC) in a Waters 2695 chromatograph with an IR Detector (2414). A CarbosepCHO 782 column operating at 75 °C with water as mobile phase (0.5 mL/min) was used for separation. A more detailed description can be found elsewhere [21].

Cell growth was monitored spectrophotometrically at a wavelength of 600 nm (DO600nm), while cell viability, total cells, and viable cells were analyzed using a Vi-Cell XR system (Beckman Coulter, Brea, CA, USA). Dry cell weight was determined gravimetrically after lyophilization. An aliquot of the medium was collected and centrifuged, and the cells were washed twice and freeze-dried. The weight of freeze-dried cells was determined and referenced to the volume of the medium collected.

The intracellular content of carotenoids was analysed using 35 mg of freeze-dried cells, as described by Moliné et al., with some modifications [22]. Cells were incubated twice in 1 mL of DMSO (with BHT as an additive at 0.05%) at 50 °C for 1 h. Subsequently, both cells and supernatants were collected by centrifugation (3000× *g*, 5 min), and 1 mL of acetone was added to the cell pellets to extract the carotenoids. The supernatant was then collected by centrifugation and mixed with the DMSO fraction. This step was repeated until total carotenoid extraction (i.e., when observing a colourless pellet). Then, 2 mL of

hexane and 0.5 mL of 20% NaCl were added to the supernatants. Finally, the organic phase was collected by centrifugation (3000× g, 10 min, 4 °C) and evaporated under a nitrogen atmosphere using a Turbo VAp LV (Zymark, Hopkinton, MA, USA). It was then dissolved in ethanol. Total carotenoid content was determined spectrophotometrically at 450 nm after appropriate dilution, and its concentration was calculated using a standard curve (0.5–10 mg/L β-carotene in ethanol with BHT 0.05%). An HPLC Agilent 1200 Series using a Diode-Array detector-UV detector at 450 nm was also used to determine the carotene profile. A C18 Agilent Poroshell 120-EC column operating at 30 °C was employed. A gradient approach of two solutions at 0.8 mL/min was used as an elution strategy. For that, Solvent A (80:20 of a methanol: aqueous solution containing 1 mM tetrabutylammonium phosphate and 1 mM propionic acid); and solvent B (60:40 of acetonitrile: methanol) were used according to the following gradient: 0–30 min 100% solvent A to 100% solvent B; 30–33 min 100% solvent B; 33–40 min 100% solvent A.

In addition, total lipid content was determined by the gravimetric method, following the method described by Sha [23] with certain modifications as detailed before [24]. Between 70–80 mg of freeze-dried cells were mixed with 3.2 mL of 4 M HCl and incubated in a bath at 55 °C for 2 h for cell lysis. After this period, 8 mL of a 2:1 (v/v) chloroform–methanol solution was added to the cells, and incubation was continued in the bath at 20 °C for 3 h for lipid extraction. After the incubation step, the solution was transferred to a glass tube for centrifugation at 2000× g for 15 min using a swinging rotor to facilitate obtaining different phases, including the organic chloroform phase with dissolved lipids. The lower phase, where the lipids are collected, was recovered in a new pre-weighed glass tube. To the remaining aqueous phase, 4 mL of chloroform was added and centrifuged again to ensure complete extraction of all lipids present in the solution with cell debris. The organic phase generated was transferred to the glass tube. Finally, the chloroform was evaporated under a nitrogen stream in a TurboVap LV evaporator (Zymark, Hopkinton, MA, USA). The tubes were incubated in a vacuum oven (Heraeus Instruments, Liedekerke, Belgium) at 40 °C until constant weight was obtained. The resulting lipid content was determined as g lipid per 100 g freeze-dried or dried cell. The lipid yield was calculated as a function of this content and its ratio to the amount of total sugars consumed by the yeasts (g lipids/g sugars consumed).

The composition of fatty acid methyl esters (FAMEs) was determined using freeze-dried cells according to the methodology described by Gallego-García et al. [20] and based on Van Wychen et al.'s methodology [25]. The method involves a transesterification process of the fatty acids present in the freeze-dried yeast (approximately 10 mg) to determine their total content in the form of fatty acid methyl esters. For this purpose, the cells were treated with HCl: methanol (0.6 M HCl [2.1% v/v] in methanol) and chloroform: methanol (2:1, v/v) solutions for cell lysis and extraction of the lipids, which were finally extracted from the methanol phase with hexane, leaving behind polar compounds such as glycerol formed after transesterification. An Agilent 7890A gas chromatograph (Santa Clara, CA, USA), equipped with a flame ionization detector and a split injector ("split" mode 1/20), and an Agilent DB-23 polysiloxane capillary column (length 30 m and 25 mm internal diameter) were used for the analysis of the fatty acids obtained. The injector and detector were operated at 250 °C and 280 °C, respectively. A 10 mg/mL solution of tridecanoic acid methyl ester (C13:0ME) was used as an internal standard to quantify the total content of fatty acid methyl esters, and a C8:0–C24:0 fatty acid calibration mixture standard was used to determine the lipid profile.

3. Results and Discussion

3.1. Potential of Biowastes as Culture Media

As listed in Table 1, a mixture of discarded vegetable residues can yield a liquid fraction after a simple crushing and centrifugation process with a total carbohydrate content of 31.3 g/L. Glucose and fructose are major sugar monomers of this stream, accounting for more than 95% of these carbohydrates. These sugar components are easily assimilated by most microorganisms, thus making these streams a promising feedstock for microbial-based processes. The production of vegetables under horticulturally intensive systems, such as greenhouse-type industries, is one of the major producers of organic waste. It is important to note that currently, agro-food residues are poorly exploited, often directly discharged into the environment and/or landfilled [14]. In addition, during its treatment, 80% of this biowaste is converted into bacterial biomass with no added value and with associated treatment and disposal costs [26]. Using agro-food biowaste as feedstock in bioprocessing will therefore benefit its sustainable management as a valuable raw material.

Table 1. Carbohydrate composition of the biowaste streams resulting from a mix of discarded vegetables (agro-food waste) and the hydrolysate obtained after pretreatment and enzymatic hydrolysis of a lignocellulosic urban pruning residue.

Carbohydrate	Agro-Food Biowaste	Concentrated Agro-Food Biowaste	Concentrated Enzymatic Hydrolysate from Lignocellulosic Urban Residue
Glucose	14.40 ± 0.04	28.54 ± 0.18	244.13 ± 3.21
Xylose	0.11 ± 0.02	0.27 ± 0.02	9.17 ± 0.02
Galactose	0.27± 0.02	0.43 ± 0.05	0.46 ± 0.02
Arabinose	0.71 ± 0.03	0.39 ± 0.04	0.16 ± 0.02
Mannose	0.16 ± 0.01	0.26 ± 0.01	0.52 ± 0.02
Fructose	15.64 ± 0.30	31.16 ± 0.47	n.d.

Values expressed in g/L. n.d., not detected.

Lignocellulosic residues are another sugar-rich source that can represent an important raw material for bioprocessing. In contrast to agro-food biowastes, lignocellulosic residues require pretreatment and enzymatic hydrolysis to obtain a sugar-rich stream or hydrolysate. As highlighted in Table 2, the water-insoluble fraction recovered after steam explosion pretreatment of a lignocellulosic urban pruning residue is made of 48% carbohydrates, considering both cellulose and hemicellulose components. After the enzymatic hydrolysis of the pretreated solid fraction and a subsequent vacuum evaporation step, a glucose-rich stream was obtained with a final total sugar concentration of about 250 g/L (Table 1). Before vacuum evaporation, a glucose concentration of around 60 g/L was obtained, corresponding to an enzymatic hydrolysis yield of about 70%. Negro et al. [19] have previously reported similar yield values during the enzymatic hydrolysis of a steam-exploded urban pruning residue obtained under similar pretreatment conditions. The use of residual lignocellulosic biomass as feedstock for SCO production has been previously investigated. Sugarcane bagasse, rice straw, or wastepaper are some examples [27–29]. Xavier et al. [28] reported the production of lipids using the hemicellulosic hydrolysate obtained after dilute acid pretreatment of sugarcane bagasse and the yeast *Lipomyces starkeyi*. The resulting lipids showed a similar fatty acid (FAMEs) profile to palm oil, commonly used in biodiesel production. Tang et al. [27] subjected rice straw to a glycerol-$FeCl_3$ pretreatment. The resulting enzymatic hydrolysate was used as media for lipid production with the yeast *Cryptococcus curvatus* ATCC 20509, obtaining 8.8 g/L of lipids with conversion yields of 0.17 g/g. On the other hand, Annamalai et al. [16] obtained 5.4 g/L of lipids using an enzymatic hydrolysate of pretreated waste office paper (5% (*w*/*v*) substrate concentration

in a 0.5% hydrogen peroxide solution; 121 °C, 30 min). Due to its high glucose content, the potential of using the lignocellulosic urban pruning residues as a carbon source for SCO production, which may contribute to the development of biorefining processes both from the economic and from the environmental point of view.

Table 2. Chemical composition of the water-insoluble solid fraction resulting from steam explosion pretreatment (190 °C, 10 min, 40 mg H_2SO_4/g raw biomass) of the lignocellulosic urban pruning residue.

Component	g/100 g of Pretreated Biomass
Glucan (Cellulose)	43.96 ± 0.44
Hemicellulose	3.74
Xylan	2.84 ± 0.14
Galactan	0.69 ± 0.05
Arabinan	0.06 ± 0.01
Mannan	0.16 ± 0.01
Acid-insoluble lignin	42.17 ± 0.81
Ash	2.41 ± 0.31

3.2. Optimization of the Pulse-Feeding Strategy for the Cultivation of R. toruloides Using Biowastes with Different Nature

Different strategies have been investigated for SCO production using oleaginous yeasts, including batch operation, fed-batch, and continuous approaches. Among them, fed-batch and continuous cultivation modes usually exhibit higher conversion yields than the batch operational mode due to improved substrate utilization [17]. A pulse-feeding strategy was designed to optimize the sugar-rich streams obtained from the agro-food waste and the lignocellulosic urban pruning residue. In addition, the yeast *R. toruloides* was selected as a microbial cell factory due to its potential to obtain carotenoids simultaneously during SCO production. *R. toruloides* was cultivated in the agro-food stream to foster cell growth. Subsequently, after sugar depletion, two pulses of the concentrated lignocellulosic hydrolysate were added to the culture to trigger lipid accumulation. Figure 1 shows the time-course cultivation process for *R. toruloides* following this pulse-feeding strategy. *R. toruloides* cells could consume both glucose and fructose within the first 24 h, reaching a cell concentration of 21.0 g/L. The sugar consumption rates decreased significantly after the first and second pulse additions. The second pulse was added after 72 h from inoculation, thus requiring 48 h for sugar depletion after the first pulse addition. In addition, a total sugar concentration of 28.3 g/L still remained in the medium after 120 h of cultivation. A final cell biomass concentration of 36.4 g/L was observed at this point. It is important to note that although the total number of viable cells remained constant after 24 h of the cultivation process, cell viability decreased from 92.8% at 24 h to 51.9% and 35.7% after 72 h (before the second pulse addition) and 120 h, respectively.

The lower sugar consumption rates and the reduction in cell viability might be indicative of cells suffering from nutrient limitation. With the aim of increasing the initial nutrient content in the system and avoiding external nutrient addition, the agro-food residue was concentrated by vacuum evaporation before inoculation until the sugar content was doubled. As can be observed in Figure 2, the use of the two-time concentrated agro-food stream improved both sugar consumption rates and cell viability within the cultivation processes. In this case, pulses were added after 24 h and 48 h, and complete sugar depletion was observed after 96 h of cultivation. Due to the higher initial sugar concentration, the cell biomass content also increased up to 32.1 g/L and 55.5 g/L after 24 h and 96 h, respectively. On the other hand, the cell viability remained above 75% until the second pulse addition (48 h) and 3–3.5 times higher number of total viable cells ($2.8–3.9 \cdot 10^9$ vs. $0.9–1.2 \cdot 10^9$ viable cells/mL) were observed, being indicative of the lower stress pressure

exerted on yeast cells, due to a lower nutrient limitation in the initial medium utilized [30]. However, cell viability decreased to 55% and 44% after 72 h and 96 h, respectively, and a similar concentration of viable cells was observed when compared to the assay using the non-concentrated agro-food waste (ca. 10^9 viable cells/mL).

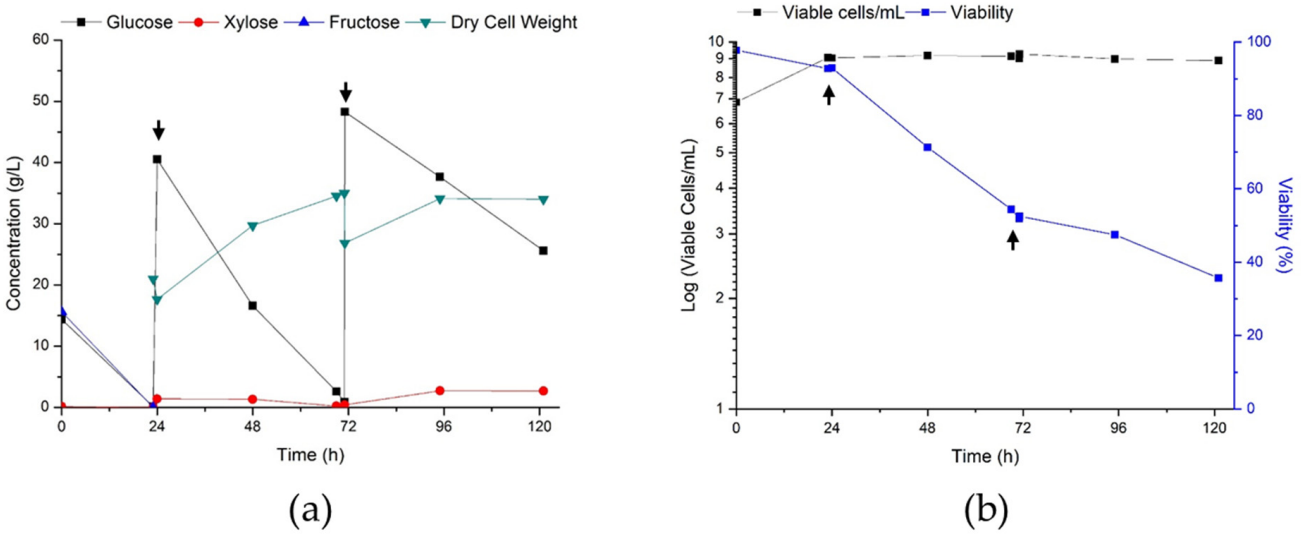

Figure 1. Time-course cultivation of *R. toruloides* ATCC 204091 in fed-batch mode using agro-food waste stream and a pulse-feeding strategy with a concentrated steam-exploded lignocellulosic urban pruning residue hydrolysate. (**a**) Sugar consumption and cell biomass production; (**b**) Cell viability and the total number of cells/mL (in logarithmic scale). Arrows are indicative of a pulse addition.

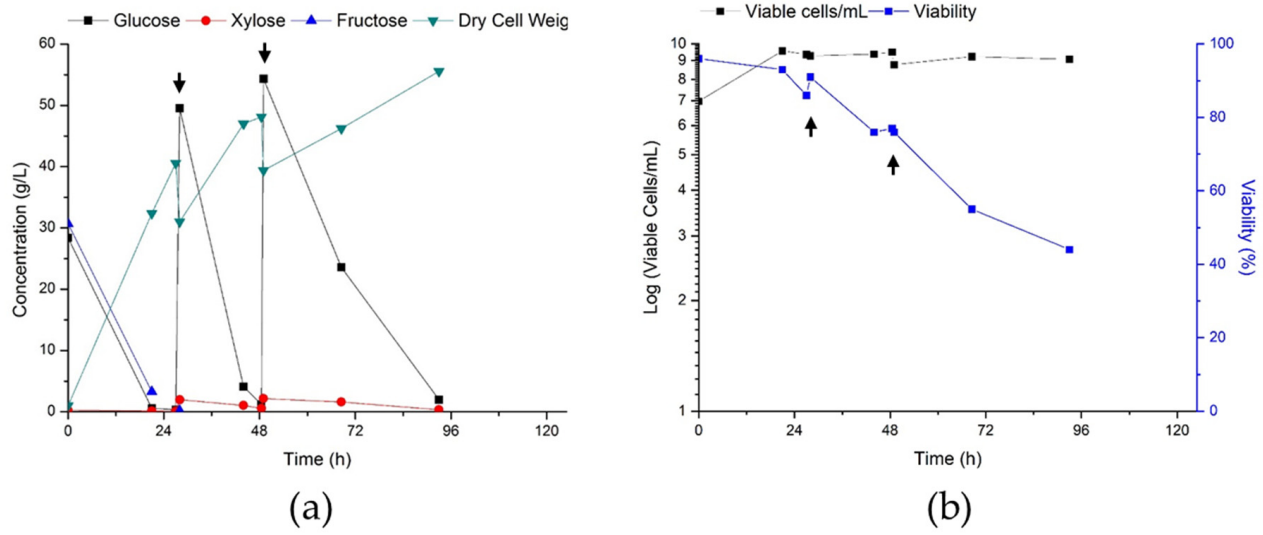

Figure 2. Time-course cultivation of *R. toruloides* ATCC 204091 in fed-batch mode using a concentrated agro-food waste stream and a pulse-feeding strategy with a concentrated steam-exploded lignocellulosic urban pruning residue hydrolysate. (**a**) Sugar consumption and cell biomass production; (**b**) Cell viability and the total number of viable cells/mL (in logarithmic scale). Arrows are indicative of a pulse addition.

Microbial growth rates are directly linked to the availability of both macronutrients and micronutrients and suitable environmental conditions [31,32]. Under nutrient limitation, microorganisms can activate specific pathways for the production of secondary metabolites, such as storage lipids, as survival mechanisms [33]. These nutrient-limiting conditions usually compromise cell growth, leading to a regulation between cell biomass formation and the secondary metabolite, and can even promote severe growth inhibition [34]. During

SCO production, cell biomass concentrations are directly related to the concentrations of the final product. Therefore, high productivity values require the production of high concentrations of cell biomass within relatively short times. The concentration of the agro-food waste stream benefits cell viability during the cultivation of *R. toruloides* ATCC 204091 under a pulse-feeding strategy using the enzymatic hydrolysate of a lignocellulosic urban pruning residue, allowing a total sugar consumption within 96 h of overall process time and a 65% increase in the final cell biomass concentration.

3.3. Effects of Media Composition on the Intracellular Accumulation of Lipids and Carotenoids

The oleaginous yeast *R. toruloides* is a promising microorganism that can co-produce both lipids and carotenoids from acetyl-CoA. As depicted in Figure 3, the designed pulse-feeding strategy allowed the accumulation of a total lipid content of 42–45% (*w*/*w*), independently of using the non-concentrated and the concentrated agro-food waste streams for the growth phase. The total amount of FAMEs showed, however, different accumulation percentages, accounting for up to 43.5% and 35.7%, respectively. In addition, lower sugar-to-lipid conversion yields were also estimated when using the concentrated agro-food waste stream (0.25 g/g vs. 0.20 g/g) (Table 3). These differences in the accumulation of fatty acids (FAs) and the conversion yields can be attributed to the lower stress exerted on yeast cells when using the concentrated growth medium, thus supporting the differences observed for the cultivation parameters. It is important to note that the higher cell biomass concentration reached allowed the final lipid concentration to increase from 16.4 g/L to 23.3 g/L. Moreover, despite the differences in the content of FAMEs, both the distribution of major groups and the individual percentage of FAs were similar when using the non-concentrated and the concentrated agro-food waste media (Figure 3, Table 3). Saturated and monounsaturated FAs represent more than 85% of the total FA content, with palmitic acid (C16:0), stearic acid (C18:0), and oleic acid (C18:1) major contributors.

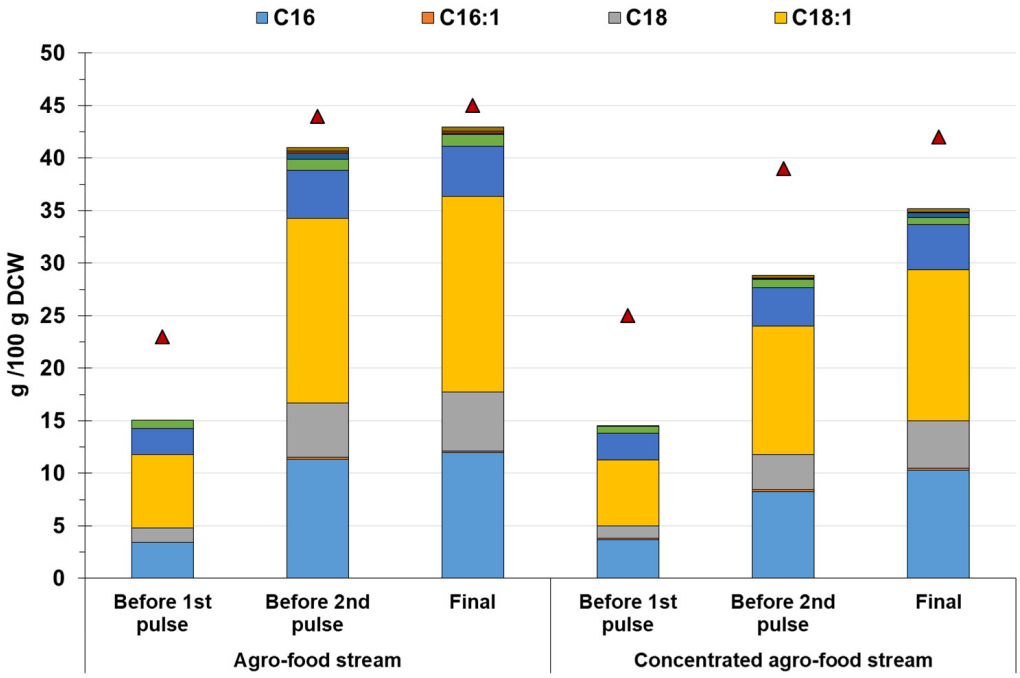

Figure 3. Total lipid production and fatty acid distribution after cultivating *R. toruloides* ATCC 204091 in the non-concentrated and concentrated agro-food waste streams as growth media following a pulse-feeding strategy with the enzymatic hydrolysate of a lignocellulosic urban pruning residue to trigger lipid accumulation.

Table 3. Lipid concentration, lipid yield, and distribution of major fatty acid methyl esters (FAMEs) groups obtained after cultivating *R. toruloides* ATCC 204091 in the non-concentrated and concentrated agro-food waste streams as growth media following a pulse-feeding strategy with the enzymatic hydrolysate of a lignocellulosic urban pruning residue to trigger lipid accumulation. SFAs: Saturated fatty acids; MFAs: Monounsaturated fatty acids; PFAs: Polyunsaturated fatty acids.

Growth Medium	Lipids (g/L)	Yield Lipids/Sugars (g/g)	SFAs (%)	MFAs (%)	PFAs (%)
Non-concentrated	16.42 ± 0.76	0.25 ± 0.02	44.42 ± 0.27	43.21 ± 0.27	13.57 ± 0.03
Concentrated	23.32 ± 1.16	0.20 ± 0.01	45.86 ± 0.10	40.96 ± 0.10	13.78 ± 1.04

The content in FAMEs is directly correlated with storage lipids. The mechanisms to increase storage lipids depend on the microbial strain. Under nitrogen limitation, the model yeast *Yarrowia lipolytica* increases its lipid pool by mainly driving the carbon source into the lipid biosynthetic pathways with minor transcriptional regulation [35]. In contrast, Mishra et al. [33] have reported a significant lipid regulation in *R. toruloides* during nitrogen starvation, supporting the hypothesis of an active lipid remodeling process in this yeast species. These authors observed a shift in the distribution of lipids from mostly phospholipids towards mostly storage lipids. Similarly, the severe nutrient-limiting conditions when using the non-concentrated agro-food waste during the growth phase resulted both in a higher content of storage lipids and lower differences between the storage lipids and the total lipids (Figure 3).

In addition to lipids, a total intracellular content of 665 and 736 μg/g DCW of carotenoids was observed during the pulse-feeding strategy using the non-concentrated and the concentrated agro-food waste, respectively (Figure 4). The highest concentrations were reached at the end of the cultivation process, which might be indicative of the benefit of having nutrient-limiting conditions to favor carotenoid accumulation. Among the different carotenoids produced by *R. toruloides*, major components considering the distribution profile are torulene > γ-carotene > torularhodin > β-carotene (Table 4). During the cultivation process, γ-carotene and β-carotene remained constant or slightly decreased, while the concentration of torulene and torularhodin increased after each pulse addition, almost doubling their concentration between the growth phase and at the end of the overall process.

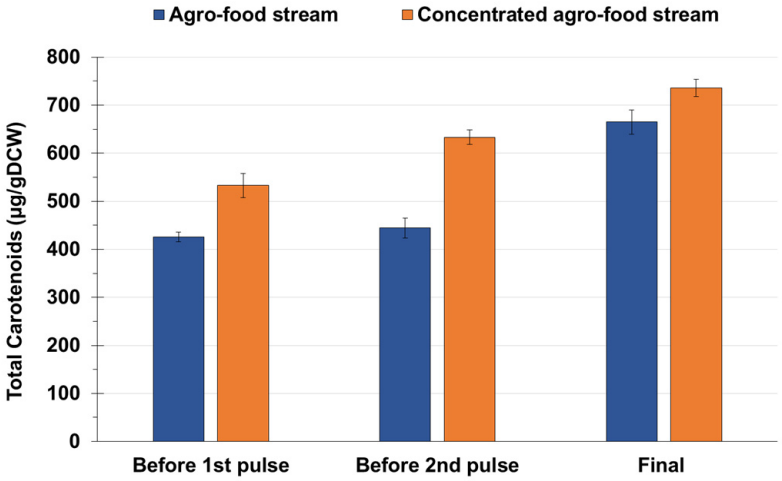

Figure 4. Intracellular carotenoid accumulation after cultivating *R. toruloides* ATCC 204091 in the non-concentrated and concentrated agro-food waste streams as growth media following a pulse-feeding strategy with the enzymatic hydrolysate of a lignocellulosic urban pruning residue to trigger lipid accumulation.

Table 4. Carotenoid profile at different time points during the cultivation of *R. toruloides* ATCC 204091 in the non-concentrated and concentrated agro-food waste streams as growth media following a pulse-feeding strategy with the enzymatic hydrolysate of a lignocellulosic urban residue to trigger lipid accumulation. Data in μg/g DCW.

Growth Medium	Process Time	Torularhodin	Torulene	γ-Carotene	β-Carotene
Agro-food waste stream	Before 1st pulse	62 ± 4	115 ± 7	109 ± 2	62 ± 8
	Before 2nd pulse	68 ± 5	141 ± 4	106 ± 6	43 ± 3
	Final	94 ± 4	289 ± 6	100 ± 8	40 ± 3
Concentrated agro-food waste stream	Before 1st pulse	72 ± 8	166 ± 6	145 ± 4	71 ± 3
	Before 2nd pulse	119 ± 5	211 ± 4	127 ± 5	61 ± 4
	Final	132 ± 16	341 ± 11	103 ± 21	59 ± 12

Microbial carotenoid production is considered a promising bioprocessing alternative to current practices. Different yeast species from the *Rhodotorula*, *Rhodosporidium*, and *Sporobolomyces* genera have been investigated for carotenoid production due to their natural capacity to produce these components. Similar total carotenoid production has been previously reported for other *R. toruloides* strains. Yang et al. [36] observed a carotenoid production of 763.85 μg/g DCW when using the UV-mutant *R. toruloides* M5 strain and optimizing the culture conditions. Sharma and Ghoshal [37] observed a total carotenoid production of 717.35 μg/g DCW with the yeast *Rhodotorula mucilaginosa* MTCC-1403 and using an acidic extract of onion peel and mung bean husk. The yeast *Sporobolomyces roseus* CFGU-S005 showed a final carotenoid production of 3.43 mg/L from a fed-batch culture and using a concentrated pasta processing waste hydrolysate as medium [38]. Higher values of carotenoid accumulation have also been reported in the literature. A total β-carotene accumulation of 1.42–2.03 mg/g DCW has been observed when cultivating the yeast *Rhodotorula glutinis* in crude glycerol and sugar cane molasses [39,40]. Thumkasem et al. [32] reported a final concentration of β-carotene as high as 2.80 mg/g DCW using the yeast *Rhodotorula paludigena* CM33 and a glucose-based synthetic medium. These authors even increased this value up to 3.25 mg/g DCW by optimizing media composition with external nutrient addition and the use of sucrose as a carbon source. In the present study, biowastes with different nature have been used for the production of both lipids and carotenoids without the need of supplementing the medium with external nutrients. These results show the great potential of this approach as bioprocessing alternative to provide added value and contribute to the better management of resources, thus reducing the associated environmental impact of waste.

4. Conclusions

Agro-food wastes and lignocellulosic urban pruning residues exhibit high potential as sugar-rich feedstock for microbial-based processing. In this context, the yeast *R. toruloides* was capable of accumulating up to 45% of its dry weight of intracellular lipids and 665 μg/g of carotenoids when using the corresponding waste-derived streams as culture media under a pulse-feeding strategy. With the aim of improving the process productivity values, the agro-food waste stream was concentrated to increase the initial nutrient concentration. This strategy allowed complete sugar consumption within 96 h without the need for external nutrient supplementation, increasing final cell biomass concentration, lipid concentration, and carotenoid concentrations up to 55.5 g/L, 23.3 g/L, and 736 μg/g, respectively. In contrast, the sugar-to-lipid conversion yields and the storage lipids percentage decreased from 0.25 g/g and 43.5% to 0.20 g/g and 35.7%, respectively, as indicative of the lower stress pressure caused on yeast cells from the nutrient-limiting conditions. Overall, these results show great potential to provide effective alternative uses to current biowaste man-

agement practices, also contributing to placing industrially relevant products with lower environmental impacts in the market.

Author Contributions: Conceptualization, M.J.N.; formal analysis, A.R.-L., J.L.F.-R. and I.B.; investigation, A.R.-L., J.L.F.-R. and I.B.; data curation, A.R.-L., M.J.N. and A.D.M.; writing—original draft preparation, M.J.N. and A.D.M.; writing—review and editing, M.J.N., I.B. and A.D.M.; supervision, M.J.N.; project administration, M.J.N.; funding acquisition, M.J.N. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by MCIN/AEI/ (10.13039/501100011033, project BIOMIO+CAR, grant number PID2020-119403RB-C22).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The original contributions presented in this study are included in the article. Further inquiries can be directed to the corresponding authors.

Acknowledgments: Fundación Cajamar (Almería, Spain) and CEDER-CIEMAT (Soria, Spain) are greatly acknowledged for providing the agro-food and the lignocellulosic urban pruning residues used in this study.

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

Abbreviations

The following abbreviations are used in this manuscript:

C/N	Carbon/nitrogen ratio
DCW	Dry cell weight
FAs	Fatty acids
FAMES	Fatty acid methyl esters
SCO	Single-cell oil

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