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Rhodotorula toruloides for carotenoid production using waste hardwood biomass

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Abstract

This work explores the potential of underutilized urban pruning residues from hardwood as feedstocks for bioprocesses based on the carotenogenic yeast *Rhodotorula toruloides*, investigating the correlation between biomass composition and carotenoids production. Enzymatic hydrolysates derived from woods and barks of sessile oak and mulberry tree were used as substrates for microbial fermentation. The results demonstrated superior titers, yields, and productivity for β-carotene and torulene compared to previously reported processes. While mulberry tree bark hydrolysate yielded the highest total sugars (16.4 g/L), sessile oak bark hydrolysate achieved the highest β-carotene production (362.7 mg/L) and yield on dry cell weight (163.4 mg/g) after 30 h of fermentation. Woody biomasses are known to contain significant amounts of extractive inhibitory compounds. Surprisingly, when these extractives were removed to promote growth, a significant drop in carotenoids titers were observed, bringing them in line with published values obtained from biomasses lacking such components. These data suggest that stress-inducing compounds present in the extractive fractions are crucial for promoting high productivity and yield, when compared with the use of biomasses lacking such components. This research highlights the untapped potential of urban woody residues, which thanks to the presence of triggering components can be considered as advantageous feedstock for microbial carotenoids production.

Keywords *Rhodotorula toruloides*, β-Carotene, Torulene, Biorefinery, Lignocellulosic biomass, Enzymatic hydrolysis

Introduction

The exploration of diverse biological feedstocks and micro-organisms is crucial for establishing efficient and environmentally friendly biorefinery and bioconversion pathways [1, 2]. Woody biomasses, derived from pruning and forestry, agricultural wastes, as well as dedicated energy crops, constitute a largely untapped resource for biorefineries due to their prevalent lignocellulosic composition. These renewable materials offer in the form of their comprised polymers cellulose, hemicelluloses, and lignins valuable alternatives to fossil resources to produce fuels, chemicals, and high-value bioproducts within the bioeconomy scenario [3–5].

The integration of microbial biotechnology into biorefinery processes can be a key factor to unlock the full valorisation potential of woody biomasses. Enzymatic hydrolysis enables the release of fermentable pentose and hexose sugars from the cellulose and hemicellulose fractions, providing carbon sources for microbial growth and biotransformations.

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Among the diverse microbial candidates for lignocellulosic biomass valorisation, the oleaginous yeast *Rhodotorula* (*Rhodosporidium*) *toruloides* has gained considerable attention. This yeast exhibits a remarkable ability to metabolize a wide range of sugars derived from lignocellulosic hydrolysates, i.e., both pentose and hexose sugars, and has demonstrated tolerance to various inhibitory compounds that can arise during biomass pre-treatment [6–10]. Furthermore, *R. toruloides* is a lipidogenic yeast and natural producer of valuable carotenoid pigments, including β-carotene and torulene. This class of compounds features significant antioxidant and provitamin A activity, and coloring properties with applications in food, feed, cosmetic, and pharmaceutical industries [7, 8, 11, 12]. The global market for carotenoids continues to grow (USD 1.48 billion in 2023, projected to grow at a CAGR of 3.5% to 2030), driven by increasing consumer demand for natural and health-promoting ingredients, as well as bio-based colorants, with β-carotene products accounting for a 24.7% revenue share in 2023 [13]. The torulene market, although smaller, is attracting an increasing interest due to its biological properties and potential health benefits [14]

Despite the growing insight into the capabilities of *R. toruloides* in the context of lignocellulosic biomass valorisation, research that specifically investigates the utilization of novel woody feedstocks from local biodiversity for carotenoid production is yet underexplored. While several studies have examined *R. toruloides* growth on conventional agricultural residues [15–20], the potential of underutilized woody forestry residues deserves further investigation. Recent studies in the literature deploying woody hydrolysate as possible substrates for *R. toruloides* are either centered on lipid rather than carotenoid production, or they report the introduction of additional carbon and/or nitrogen sources [21–23]. In addition, most of the recent publications on the topic are focused on the valorisation of by-products from pruning, mainly of olive trees [24–26] or vines [27–29], whereas urban pruning residues are exploited mainly for energy or chemical production via physical and/or chemical treatments [30–32]. Thus, there is a lack of studies on the valorisation of such urban residues by microbial action, beyond composting or anaerobic digestion [4, 30, 31] To contrast this use in primarily low-value applications, this study aims to provide alternative valorisation for these lignocellulosic biomasses of urban origin to obtain a product connected to a higher value, i.e., carotenoids.

This study aims consequently at addressing the potential of urban pruning/forestry residues in the form of sessile oak (**SO**, *Quercus petraea*) and mulberry tree (**MT**, *Morus alba*) biomasses, sourced from an urban park within the Milan metropolitan area, as substrates for *R. toruloides* growth and carotenoid production.

The initial steps of this work involve the hydrolysis of the residual biomasses to obtain growth media for the yeast, followed by the analysis of the resulting sugar and nitrogen concentrations from both wood (**W**) and bark (**B**) of sessile oak and mulberry tree, i.e., **SOW**, **SOB**, **MTW**, and **MTB**. Furthermore, the woody hydrolysates were presented to *R. toruloides* in a separated hydrolysis and fermentation (SHF) setup. Differences in carotenoid production during fermentation time were analyzed, with particular attention to identify components of the woody biomass that might trigger or inhibit carotenoid biosynthesis.

Materials and methods

Chemicals

All chemicals used were analytical grade. Reagent grade water was provided by a Milli-Q® Integral 5 purification system (Merck KGaA, Darmstadt, Germany). Dichloromethane (DCM), toluene (tol), ethanol (EtOH), ethyl acetate (AcOEt) and acetone (GC–MS grade) were purchased from Carlo Erba (Cornaredo, Italy). Sulphuric acid (H_2SO_4) (95–97%) was obtained from Merck KGaA (Darmstadt, Germany). Acetonitrile (LC–MS grade) was supplied from VWR International SrL (Milan, Italy). Standards of β-carotene (97% purity) and caffeine (99% purity) were obtained from Sigma-Aldrich (St Louis, MO, USA), while the standard of torulene (> 95% purity) was provided by CaroteNature (Münsingen, Switzerland).

Biomass collection and preparation

The residual woody biomasses used in this study are sessile oak (*Quercus petraea*) wood (**SOW**) and bark (**SOB**), mulberry tree (*Morus alba*) wood (**MTW**) and bark (**MTB**). These materials were collected as leftovers from the maintenance of Parco della Besozza (Pioltello), an urban park in the metropolitan area of Milan (Italy). These biomasses were used as obtained, e.g., in the form a conventional urban pruning/forestry workflow that produced them in an only roughly separated form between bark and wood. Consequently, both starting materials mutually contain residues of the other component, i.e., bark or wood. Importantly, these biomasses were applied in this study without any upstream detoxification step, yielding the possibility to explore eventual inhibiting factors. The biomasses were dried and stored at room temperature till processing. Woody biomasses were mechanically grounded to a final particle size of less than 1 mm. The resulting powdered biomass was used for all the steps of treatment and analysis.

Microbial strain, media, and growth conditions

Rhodotorula (Rhodosporidium) toruloides DSM 4444 was obtained from DSMZ (German Collection of Micro-organisms and Cell Cultures, GmbH) and kept in cryotubes at − 80 °C in 20% (v/v) glycerol. The composition of the medium (YPD) for the pre-inoculum was as follows: 20 g/L peptone, 10 g/L yeast extract, 20 g/L glucose. Yeast extract and peptone were purchased from Biolife Italia S.r.l. (Milan, Italy). All other reagents were purchased from Sigma-Aldrich Co. (St Louis, MO, USA). After plating on YPD, a pre-inoculum was run in rich medium until the stationary phase. Then, cells were inoculated at 0.25 OD in 250 mL shake flasks with 50 mL medium volume at 30 °C and 160 rpm in SHF setting using woody hydrolysates without any supplementation. Growth was measured in terms of optical density (OD) at 600 nm. Samples (1 mL) were collected during the growth and centrifuged (8000 rpm, 5 min). The supernatant was used for sugar quantification, whereas the cellular pellet was used for carotenoid extraction.

Enzymatic hydrolysis of woody biomasses

Enzymatic hydrolysis of the woody biomasses was performed using the enzyme cocktail NS22119, kindly provided by Novonesis (Novonesis A/S, Copenhagen, Denmark). NS22119 contains a wide range of carbohydrases, including cellulase, arabinase, pectinase, β-glucanase, hemicellulase, and xylanase from *Aspergillus aculeatus*, as described by the producer. As a first step, 40 mL of 15% of biomass (w/v) was autoclaved in closed bottles at 121 °C for 1 h to both sterilize and mildly pre-treat the biomass. Additional pre-treatment steps were not considered to promote the development of a simpler overall process and to test the ability of the enzymatic cocktail to withstand harsher accessibility to the sugar components. Afterward, the NS22119 enzyme mix (11.9% w/$w_{biomass}$) was added to the biomass (with a natural initial pH of around 5.5, measured by litmus test) and incubated at 50 °C in a water bath under mild agitation (105 rpm) for 6 h. At the end of the process, the hydrolysates were centrifuged (8000 rpm, 10 min) to precipitate the insoluble components and collect the supernatant to be used as growth medium for *R. toruloides*.

Characterization of woody hydrolysate using dinitrosalicylic (DNS) test

The dinitrosalicylic (DNS) test was used to determine the total reducing sugar content of the obtained hydrolyzed. An optimized and miniaturized DNS protocol was deployed [33]. DNS reagent consists of: 10 g/L 3,5-dinitrosalicylic acid (Sigma-Aldrich), 16 g/L NaOH (Merck), 30 g/L potassium sodium tartrate (Carlo Erba). For quantification, a glucose calibration curve (30–0.6 g/L) was prepared. In each tube, 190 µL of the DNS reagent and 10 µL of sample were inserted and then vortexed. The tube was heated at 100 °C for 2 min and then cooled to room temperature. Following heat treatment, all samples were transferred to 96-well plates and analyzed at 531 nm in a multiplate reader (VICTOR X3, PerkinElmer). Specific sugars (glucose, fructose, sucrose, xylose, and galactose) were quantified using the spectrophotometric enzymatic kits K-SUFRG, K-XYLOSE, and K-ARGA (Megazyme, Southern Cross Rd, Bray, Co. Wicklow, Ireland), following the manufacturer's instructions. For this purpose, samples were treated by the addition of 20 g/L of polyvinylpolypyrrolidone (PVPP), shaken vigorously for 5 min and then filtered. Nitrogen content in the form of primary amines was quantified using the PANOPA enzymatic kit (Megazyme).

Intracellular carotenoid's extraction

Acetone was used for extracting carotenoids from *R. toruloides* cells as described elsewhere (Bertacchi et al. 2020). In brief, 1 mL of cells was collected and harvested by centrifugation at 10000 rpm for 7 min, and the pellet was then resuspended in 1 mL acetone and broken using glass beads by thorough agitation with a FastPrep-24™ (MP Biomedicals, LLC, Santa Ana, CA, USA) with three cycles of 30 s each at 4 °C. Carotenoids were thus extracted in the acetone phase, collected in the supernatant by centrifugation and stored at − 20 °C. The extraction was repeated with a new aliquot of acetone until the biomass turned colorless.

Scanning electron microscope (SEM) analysis of grounded biomasses

SEM–EDS observations were performed at the Platform of Microscopy of the University of Milano-Bicocca (PMiB) with a field emission gun (FEG) SEM Zeiss Gemini 500, operating at 3 and 5 keV and equipped with a Bruker QUANTAX integrated wave-dispersive/energy-dispersive (WDS/EDS) system. Metallization was carried out using an Edwards Sputter Coater S150B, with Au/Pd target (70% Au/30% Pd) in Ar atmosphere, current of 40 mA, voltage 1 kV, sputtering time 1 min, and depositing a 10 nm thick Au/Pd layer.

Removal of extractives from grounded biomasses

Wood or bark powder was placed into a crucible glass filter, pore size III, for subsequent solvent extraction using a standard 100 mL Soxhlet extractor. The first extraction utilized 150 mL of dichloromethane (DCM) for 6 h, followed by a second extraction using 150 mL of

a 1:1 (v/v) toluene/ethanol (tol/EtOH) mixture for 12 h. The final extraction used 150 mL of acetone for 6 h. The extracts were subsequently isolated by removing the solvents in vacuo.

Determination of acid-insoluble and acid-soluble lignin content through Klason extraction

For the determination of acid-insoluble lignin, a NREL procedure was adopted [35]. Approximately 100 mg of milled wood or bark was carefully weighed into a test tube. To this, 2 mL of 72% (w/v) (H_2SO_4) solution was added at room temperature, with intermittent stirring over a 2 h. The resulting solution was then diluted with deionized water to achieve a 3% (w/v) H_2SO_4 concentration. Then, the solution was transferred to a sealed flask and subjected to heating at 120 °C and 2 bar for 7 min using an autoclave (LaborAutoklav, Certoclav, Austria). The solution was filtered through a crucible of size M, washed with deionized water until a neutral pH was attained, and the crucibles were placed in an oven at 105 °C for 12 h to fully dry the sample. The determination of acid-insoluble lignin was performed gravimetrically.

For the assessment of acid-soluble lignin, the filtrate obtained from the acid-insoluble lignin procedure was diluted to 100 mL using deionized water. The quantification of acid-soluble lignin was performed by calculating UV absorbance at 205 nm, utilizing an extinction factor of 113 L/g·cm, which was chosen according to literature for hardwoods [35].

Characterization of woody hydrolysate using ^{1}H nuclear magnetic resonance (NMR) spectroscopy

To a 500 µL aliquot of the hydrolysate solution were added 100 µL D_2O as lock-triggering solvent. A Bruker 400 MHz spectrometer controlled via TopSpin 4.1.4 with a 5 mm double resonance broadband inverse probe was used to acquire ^{1}H NMR spectra at 30 °C. The Bruker zg pulse sequence was used with NS = 48, applying water signal suppression. MestreNova Version 9.0.1 (Mestrelab Research S.L.) was used for data processing.

Characterization of woody hydrolysate using ^{1}H–^{13}C heteronuclear single quantum coherence (HSQC) analysis

To a 500 µL aliquot of the hydrolysate solution 100 µL D_2O were added as lock-triggering solvent. A Bruker 400 MHz spectrometer controlled via TopSpin 4.1.4 with a 5 mm double resonance broadband inverse probe was used to acquire HSQC spectra at 30 °C. The Bruker hsqcetg pulse program in the DQD acquisition mode was applied with NS = 64; TD = 2048 (F2) and 512 (F1); SQ = 12.9869 ppm (F2) and 164.9996 ppm (F1); O2 (F2) = 2601.36 Hz and O1 (F1) = 7799.05 Hz; D1 = 2 s; CNST2 ^{1}J(C–H) = 145; and acquisition time F2 channel = 197.0176 ms and F1 channel = 15.4164 ms. For each sample, the pulse length of the 90° P1 high-power pulse was optimized. MestreNova Version 9.0.1 (Mestrelab Research S.L.) was used for data processing.

Characterization of wood and bark extracts using gas chromatography coupled with mass spectrometry (GC/MS)

Gas Chromatographic–mass spectrometric analyses of the dried extractives were performed by re-dissolving a dried sample of the extracts in 500 µL of dichloromethane. The chromatographic separations were performed using a Shimadzu GCMS QP2020NX (Shimadzu Corporation, Kyoto, Japan) equipped with Shimadzu autosampler AOC20i. An SH-Rxi-5 ms fused silica capillary column [stationary phase (5%-phenyl)-methylpolysiloxane, 30 m × 0.25 mm i.d., 0.25 µm, Shimadzu Corporation, Kyoto, Japan] was used as the stationary phase and helium (UHP grade) as the carrier gas. The system was operated in 'linear velocity mode' with a starting pressure of 100 kPa, 280 °C injection temperature, and 280 °C interface temperature. The injection volume was 2 µL, and the injection port operated in splitless mode. The temperature program was set as follows: the initial temperature of 50 °C was held for 1 min, then increased at a rate of 10 °C/min to 280 °C, which was maintained for 15 min. The MS operated in electron ionization mode (EI) at 70 eV, acquiring in full-scan mode in the *m/z* range of 50–500. LabSolutions–GCMS Version 4.54 software (Shimadzu Corporation) was used for system control, instrument management, and data acquisition. Substances were identified using NIST MS Search, version 2.4 (2020) (National Institute of Standards and Technology, Gaithersburg, MD, USA).

Carotenoids quantification using flow injection analysis coupled by mass spectrometry (FIA/MS)

Following acetone-based extraction of carotenoids described in "Intracellular carotenoid's extraction" section of the "Materials and methods", quantitative analysis of β-carotene and torulene was performed on the resulting extracts. Indeed, β-carotene and torulene are the main carotenoids obtained from *R. toruloides* growth on residual biomasses and acetone extraction, compared to other species that could be synthetized (e.g., torularhodin) [36]. β-Carotene and torulene were used as analytical standard and caffeine as internal standard (10 ppm) to construct five-point calibration curves (concentration range 0.01–1 ppm for torulene, 0.5–20 ppm for β-carotene). The calibration curves were obtained in flow injection analysis (FIA) mode, which was applied for the analysis of the extracts in acetone as well, previously centrifuged at 1500 rpm for 5 min. A binary LC 20AT pump, coupled with an SPD M20A UV system

and a 2010 EV single-quadrupole mass spectrometer equipped with an electro-spray ionization (ESI) source was used. All modules are Shimadzu Corporation (Kyoto, Japan). The mobile phase was set at 100% acetonitrile with a flow rate of 0.3 mL/min; injection volume was 20 µL. The operating conditions of the ESI–MS system were as follows: nebulizer gas (N_2, purity > 98%) with flow rate of 1.5 mL/min, curtain desolvation line (CDL) at 240 °C and heat block (HB) at 230 °C, capillary voltage 2.5 kV. Acquisition of MS spectra was performed in positive ion mode in selected ion monitoring (SIM), observing fragments at *m/z* 195 for caffeine, 534 for torulene, and 536 for β-carotene. LCMS solution Version 3.30 268 software (2002–2005; Shimadzu Corporation) was used for instrument management and data acquisition.

Determination of yields and C/N ratio

"Yield of carotenoids on initial biomass" was calculated as the ratio between the carotenoids production and the amount of biomass used in the enzymatic hydrolysis. Similarly, "yield on hydrolysate sugars" or "yield on consumed sugars" has as denominator the amount of sugars in the hydrolysate provided to cells as growth medium or the amount of sugars effectively consumed after 30 h of growth, respectively. "Carotenoids yield (mg/g)" was calculated by dividing carotenoids production with the dry cell weight (DCW). DCW was calculated by multiplying the measured OD with the conversion factor 0.43. This factor was obtained from previous work on *R. toruloides* producing carotenoids growing using residual biomass hydrolysate as the ratio between the obtained ODs and DCW [37]. C/N ratio of hydrolysate was calculated as the ratio between the carbon content in the sugars measured by DNS assay and the measured primary nitrogen source. The carbon content was calculated by multiplying the total reducing sugars amount by 0.4, accounting for 40% carbon content in glucose and fructose, representing the majority of the sugars contained.

Statistical analysis

Values shown are the means ± SD of three independent experiments. For statistical analysis, a heteroscedastic two-tailed *t* test was applied, and the *p* value is reported ($p < 0.05$).

Results and discussion

Characterization of woody biomass

The complex nature of residual woody biomass is a well-known initial hurdle in defining its application in a microbial-based biorefinery [1, 38, 39]. In addition, the intrinsic biodiversity, in terms of plant species and parts, is key to highlight differences in carotenoids production possible arising from the procedure steps as hydrolysis and fermentation (SHF). For these reasons, the composition of the biomass selected for this study, i.e., sessile oak wood (**SOW**) and bark (**SOB**), as well as mulberry tree wood (**MTW**) and bark (**MTB**) was initially characterized. Table 1 summarizes the main components constituting these lignocellulosic biomasses, in terms of the sugar fraction vs. the lignin content and considering the extractable compounds from the three solvent consecutive extractions.

Overall, the delineated compositions are in line with what is expected from hardwoods, with less cellulose contents in the barks [40]. Bulk sugar contents between wood and bark feedstock are not statistically different. Comparing woods and barks across the two plant species **SO** and **MT**, their extractives are the only components indicating a significant difference between the two biomass types ($p < 0.05$).

The analysis of the lignin content of the various biomasses (Table 1) shows values that seem high for hardwood species. It can be assumed, based on literature data [41] that a low percentage of the determined insoluble Klason lignin content is actually comprised of humins, known to form upon sugar degradation during Klason analysis and co-precipitating with the insoluble lignin. Interesting is the fact that in the barks is found more lignin than in the bulk. This must be explained by the residual wood in the bulk bark material and the presence of suberins that behave eventually like the lignin in the applied NREL procedure [42]

Detailed analysis of the various extractives obtained was performed using GC/MS to provide an in-depth characterization of their composition. Indeed, rather

Table 1 Composition of biomasses used in this study

Biomass component	SOW	SOB	MTW	MTB
Cellulose and hemicellulose [% (w/w)]	61 ± 11	55 ± 5	59 ± 9	53 ± 5
Lignin (acid soluble) [% (w/w)]	4 ± 1	3.7 ± 0.8	1.5 ± 0.1	2.8 ± 0.6
Lignin (acid insoluble) [% (w/w)]	28 ± 3	31 ± 2	32 ± 3	35 ± 2
Total extractives [% (w/w)]	6.4 ± 0.2	10.5 ± 0.3	7.4 ± 0.4	8.9 ± 0.8

Cellulose and hemicellulose [% (w/w)] are calculated as the difference of the sum of lignin (acid soluble), lignin (acid insoluble) and total extractives. Total extractives [% (w/w)] is given by the sum of DCM, EtOH/tol and acetone consecutive extractions

scant information is reported in the literature on the composition of the trees, wood and bark under study. The results, detailed in Additional file 1: Table S1, show the expectable range of bioactive small molecules, with a slightly richer composition found for two bark samples, i.e., **SOB** and **MTB**. Several molecular classes of compounds have been identified in the extracts, mostly fatty acids and sterols, but also marker species belonging to other species. Mulberry tree resulted to be richer in extractives than sessile oak, showing high amount and variety of fatty acids, phenolic compounds, vitamins and coumarins especially in the bark. The three isomers of tocopherol, i.e., δ-tocopherol, γ-tocopherol, α-tocopherol, all antioxidant compounds known as components in vitamin E, have been already observed in mulberry fruit and bark [43]. Coumarins were found especially in leaves [44]. Umbelliferone, methyl ostruthin, ostruthin, and esculetin, found now in this study, are also typical for the *umbelliferae* family of plants. With respect to sessile oak, the **SOW** profile is particularly poor, while **SOB** interestingly presents the wider variety of terpenoids with respect to the other samples. The identified terpenes belong to the family of diterpenes (copalol), tritepenes (β-amyrone, 24-norursa-3,12-diene, lupeol, β-amyrin, glutinol, copalol and simiarenol) and sesquiterpenes (humulenol-II). **SOB** extractives show also the unique presence of alcohols, alkenes and other compounds, such as benzaldehyde and benzaldehyde diethylacetal, compared to the other biomasses.

Enzymatic hydrolysis of woody biomasses

To unlock the potential of lignocellulosic biomasses in microbial-based biorefineries, an enzymatic cocktail able to release both hexose and pentose into the liquid phase sugars is commonly used. As described in the "Enzymatic hydrolysis of woody biomasses" section, powdered biomasses, i.e., **SOW**, **SOB**, **MTW**, and **MTB**, were subjected to enzymatic hydrolysis. Data reported in Fig. 1A clearly show the effect of the action of such an enzymatic cocktail in terms of total reducing sugars, with an increase in the carbohydrates released from the sole pre-treatment.

Specifically, given the same initial amount of biomass processed per volume [15% (w/v)], data show that **MTB** is the raw material providing the highest amount of released sugars (16.4 ± 0.9 g/L), whereas on **MTW** basis the lowest content is obtained, reaching approx. only 50% of that seen for the **MTB** sample (8.3 ± 0.5 g/L) ($p < 0.05$). Interestingly, considering the yield of enzymatic hydrolysis on the sugar released by the simple pre-treatment, the wood moiety provided consistently higher values, with a 16-fold increase for both **SOW** and **MTW**, compared to the correspondent barks showing a 3.2 and ninefold increase for **SOB** and **MTB**, respectively. This fact can be related to the different structural characteristics of bark with respect to wood. Scanning electron microscopy (SEM) images collected on the various biomasses after grounding show that the bark samples seem slightly more porous, which should expose a larger surface for the enzymes to work on (Fig. 2). Nevertheless, the data collected are consistent with previous research suggesting a negative impact of bark presence toward enzymatic saccharification, using as biomass source residues of spruce, birch, and Douglas Fir [45, 46]

To investigate the composition of the carbohydrate mixture in the hydrolysates of the various biomasses, $^{1}H–^{13}C$ HSQC analyses were performed alongside quantifications by enzymatic assays. Tables 2 and 3 report the results obtained in the assays, whereas Fig. 3

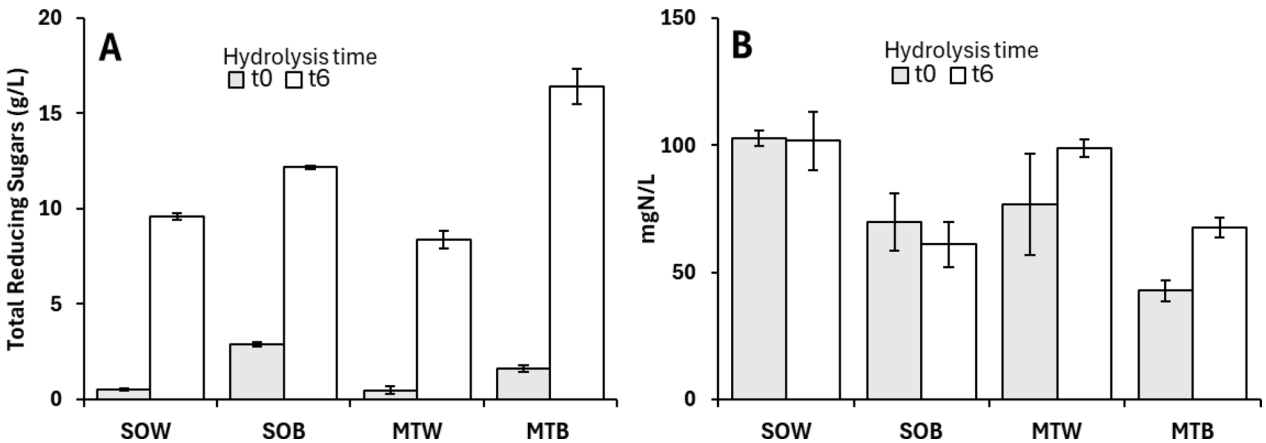

Fig. 1 Total reducing sugars released by enzymatic hydrolysis of the different woody biomasses (**A**) and corresponding primary nitrogen sources (**B**). Values are the means ± SD of three independent experiments

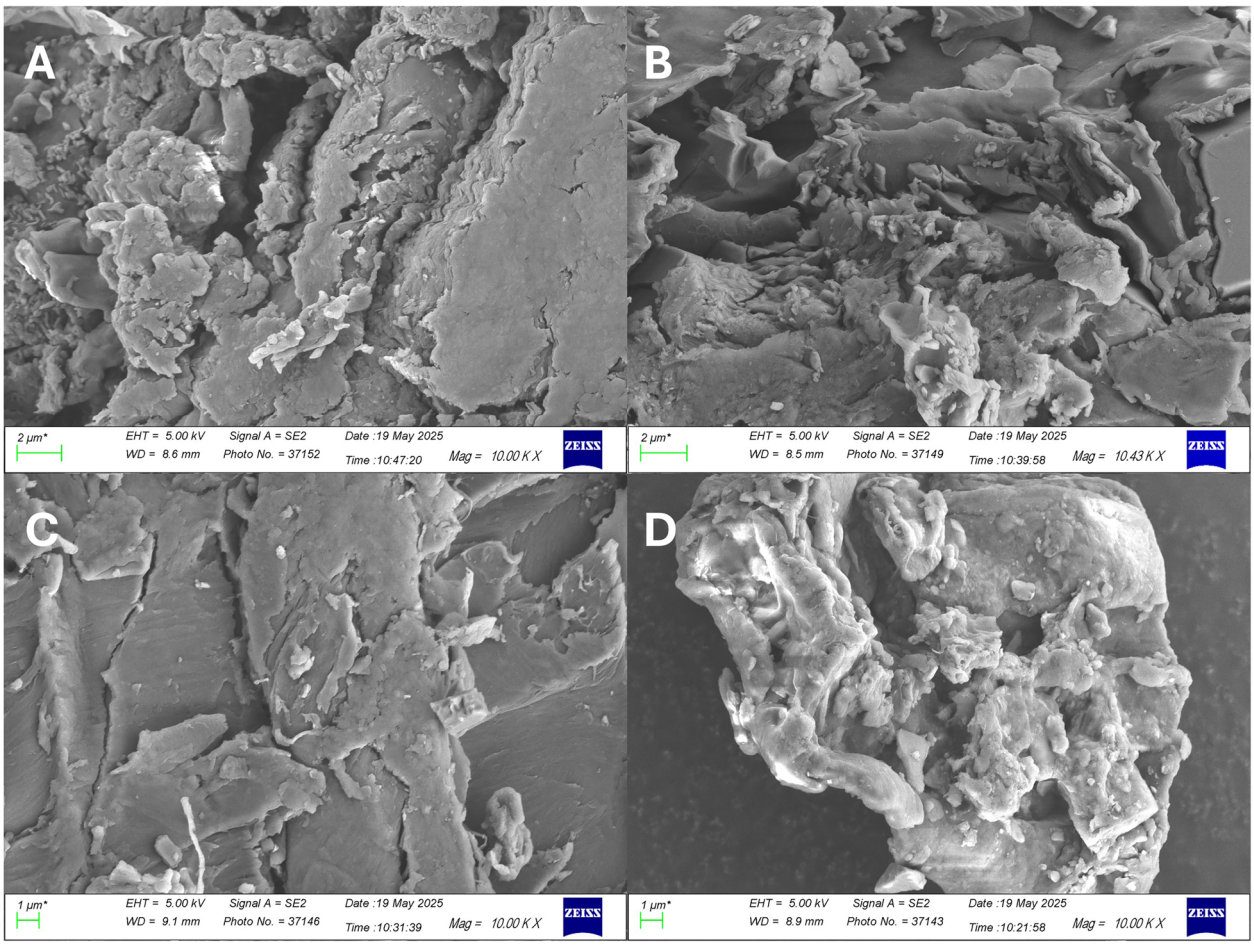

Fig. 2 SEM images of grounded biomass particles: **SOW (A)**; **SOB (B)**; **MTW (C)**; and **MTB (D)**

shows the HSQC spectra obtained for the various hydrolysates.

The wet-chemical analysis (Table 3) reveals the presence of glucose, fructose, xylose, and galactose, indicating the successful hydrolysis, especially of the hemicellulose component of the biomasses studied. The carbohydrates identified are in line with previous findings regarding oak-based hemicelluloses [47–49]. These results are confirmed by the HSQC analyses, with the interesting exception of the xylose component in **SO** samples. While the wet-chemical test shows higher quantities for xylose in **SO** samples than in **MT** samples, HSQC signal intensity suggests the contrary. The reason for this cannot be delineated precisely based on the data available. More importantly, however, HSQC analyses were able to unveil the presence of additional carbohydrates and derivatives that the wet-chemical test could not target. Via a series of characteristic cross-peaks assigned based on various literature sources [48, 50–58], essentially the same species were identified in all the samples, with variations in relative abundances. **SO**-derived samples show in HSQC analyses only traces of xylose and methyl glucuronic acid. **MT** samples show in HSQC analyses traces of arabinose, whereas this carbohydrate is not clearly detectable in **SO** samples. Acetylated carbohydrates have been identified in the form of acetylated xylose in all samples. This is indicative of the expectable presence of acetylated xylan moieties, especially in **MT** samples, and thus in line with one of the most famous representatives of hardwood hemicellulose features.

HSQC spectra indicate also the presence of alkanoates, originating eventually from waxes present in the hydrolysate. Other weak cross-peaks could be assigned to alkyl residues, eventually stemming from alkylated sugars. Most importantly, **SO**-derived hydrolysates **SOW** and **SOB** contain very weak cross-peaks in the aromatic region that could potentially indicate the presence of pyrogallyl and gallate units, as such presence in tannins, with the latter especially in the typically oak-derived tannic acid. The cross-peak typical for a gallate unit is more pronounced in the **SOB** sample,

Table 2 Carbohydrates and inhibitors identified in hydrolysates after 6 h of the biomasses used in this study by means of $^{1}H–^{13}C$ HSQC analyses (*HSQC*) and a full list of identified signals and their respective shifts is given in Additional file 1: Table S1

Carbohydrate	*colour code*	SOW	SOB	MTW	MTB
Arabinose		---	---	traces	traces
Fructose		X	X	X	X
Galactose		X	X	X	X
Glucose		X	X	X	X
Mannose		X	X	X	X
Methyl gluconoric acid		traces	traces	traces	traces
Xylose		traces	traces	X	X
Other compounds					
Alkanoyl residues		X	X	X	X
Alkyl residues		traces	traces	traces	traces
Gallate residues		X	X	traces	traces
Pyrogalloyl residues		X	X	X	X

Table 3 Monosaccharides measured by specific enzymatic assays in hydrolysates after 6 h of the biomasses used in this study by means of tested enzymatic assays

Titer (g/L)	SOW	SOB	MTW	MTB
Glucose	2.4 ± 0.1	1.5 ± 0.1	3.4 ± 0.4	5.3 ± 1.7
Fructose	2.2 ± 0.1	2.8 ± 0.1	2.5 ± 1.3	4.0 ± 1.0
Xylose	1.3 ± 0.2	1.0 ± 0.1	0.6 ± 0.1	0.8 ± 0.1
Galactose	1.0 ± 0.1	0.7 ± 0.2	0.7 ± 0.2	0.6 ± 0.2

Values are the means ± SD of three independent experiments

whereas here the pyrogallyl peak is hardly detectable. This is a relevant observation, since propyl gallate was demonstrated to increase microbial carotenogenesis in the thraustochytrids [59]

The combined NMR analyses do not indicate the presence of lipids, since typical glycerol-derived cross-peaks are absent. Neither amino acids nor peptide residues were detected. Samples do contain nitrogen sources, however, as revealed by wet-chemical tests, but contents in amino acids are supposedly too low as that they would exceed the detection threshold of around 1–2% (w/w) typical for NMR analyses. Figure 1B shows the amount of primary nitrogen source found in the hydrolysate. Nitrogen content is not increased by the action of saccharifying enzymes, with MTB as an exception. The measured amount of nitrogen permits to calculate the C/N ratio for the different hydrolysates, being 38 for **SOW**, 80 for **SOB**, 34 for **MTW**, and 97 for **MTB**. C/N ratio is one of the factors involved in triggering the production of lipids and carotenoids in oleaginous yeasts [7] Given the presence of both carbon and nitrogen sources, the ability of *R. toruloides* to grow in such media without any supplementation of nutrients, or elimination of possible toxic compounds was directly tested, to further improve the sustainability aspects of the present work in respect to published bioprocesses based on this microbial cell factory and residual biomasses (Table 4).

R. toruloides production of carotenoids from woody hydrolysate

After confirming the presence of fermentable sugars in the hydrolysates, the ability of *R. toruloides* to grow in such media, withstanding possible inhibitory compounds, and to produce carotenoids was tested. As described in "Microbial strain, media, and growth conditions" section, the yeast was directly inoculated into the woody hydrolysate. Growth, sugar consumption, and carotenoid production were monitored through periodic sampling. The initial total solid loading of the biomasses was not adjusted to normalize sugar concentrations in the media; instead, experiments started from the same total solid loading, as such providing a comparison of the overall saccharification and fermentation (SHF) processes. Figure 4A–D summarizes the results obtained from the growth kinetics on **SOW**, **SOB**, **MTW**, and **MTB** hydrolysates.

MTB is the biomass supporting the highest growth in terms of OD (Fig. 4D) ($p < 0.05$), which is consistent with having the highest initial sugar content among the hydrolysates (Fig. 1A). While *R. toruloides* shows a similar growth profile in **MTW** and **SOW** (Fig. 4A, C) considering the initial difference in the provided sugars, growth in **SOB** hydrolysate resulted to be linear until reaching a plateau (Fig. 4D), rather than being exponential. Since the NMR analysis of **SOB** revealed the presence of substructures typical for tannins, above the NMR detection threshold, which is typically around 5% (w/w); it could be speculated that this tannin presence is interfering with yeast productivity. Similarly, terpenes detected in **SOB** extractives in higher concentration and variety with respect to the other samples by GC/MS analysis (Additional file 1: Table S1), could have an inhibitory effect.

Nevertheless, *R. toruloides* was able to grow in all cases, consumed the available carbohydrates, despite not completely, and, most importantly, accumulated carotenoids. Specifically, both β-carotene and torulene were detected intracellularly, with the first being the more abundant of the two. The ratio between the two carotenoids in oleaginous yeasts may vary depending

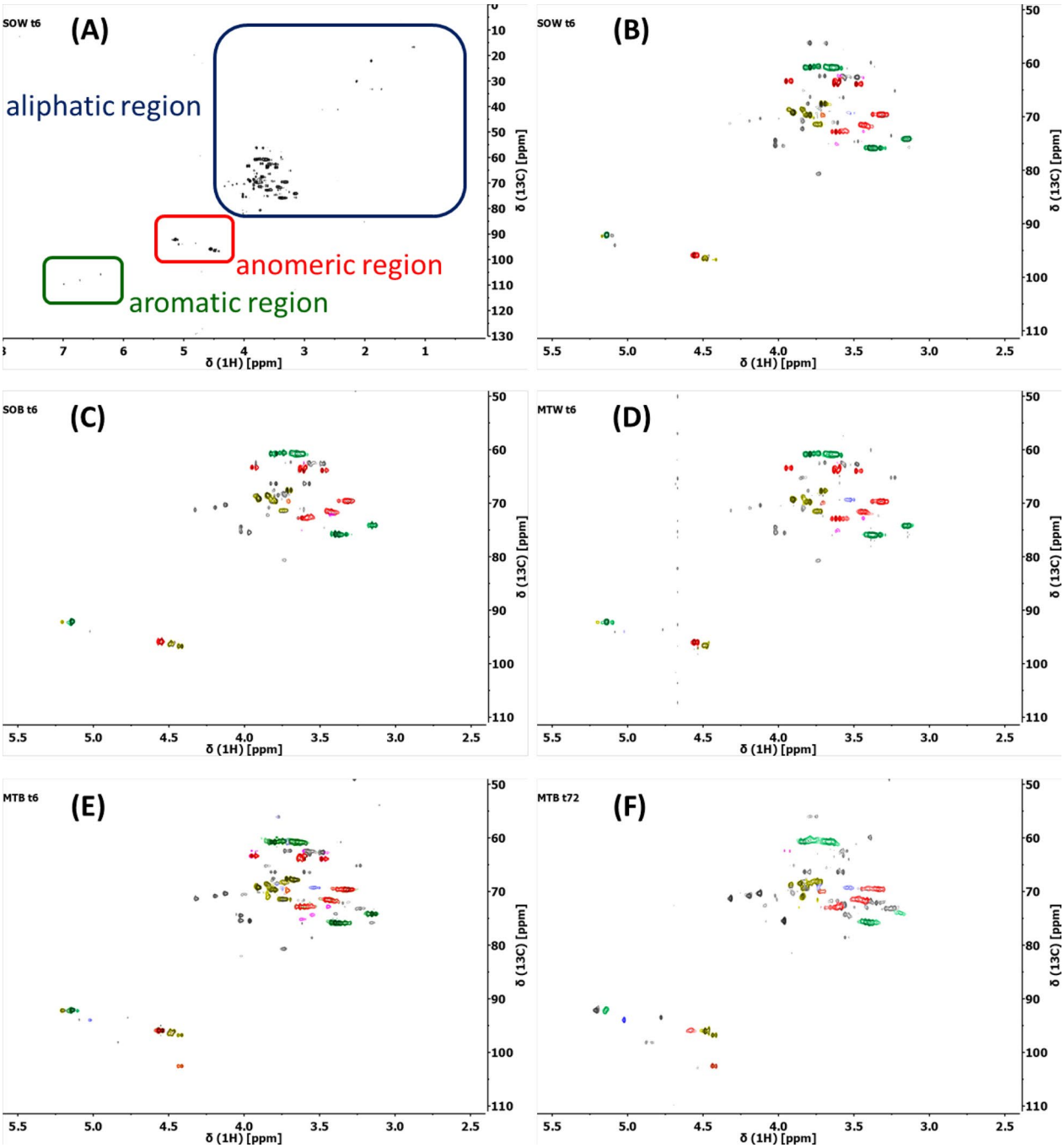

Fig. 3 ^{1}H–^{13}C HSQC spectra of **SOW** full spectra (**A**); **SOW** partial aliphatic and numeric region (**B**); **SOB** partial aliphatic and numeric region (**C**); **MTW** partial aliphatic and numeric region (**D**); **MTB** partial aliphatic and numeric region (**E**); and **MTB** partial aliphatic and numeric region after 72 h (**F**)

not only on strain, but also on the conditions, such as sugar availability, C/N ratio, or light exposure [60–63]. The highest production of β-carotene occurred, considering the above discussed inhibitors, interestingly on **SOB** hydrolysate after 30 h of growth, reaching 362.7 ± 33.9 mg/L (Fig. 4B), followed by **MTB** with 165.3 ± 22.6 mg/L of β-carotene (after 30 h of growth) (Fig. 4D). The production of torulene was instead observed to be similar across the provided biomass around 24 and 30 h of growth, ranging from 4.5 to

Table 4 Examples from literature for the production of carotenoids from residual biomasses employing *R. toruloides* strains, with a focus on the comparison between productivities and yields on DCW obtained

R. toruloides strain	Biomass	Treatment	Added N source	Starting sugars (g/L)	Time (h)	Carotenoid production (mg/L)	Carotenoids yield (mg/g DCW)	Carotenoid productivity (mg/L*h)	References
DSM 4444	Defatted waste wheat bran	Biological (Cellic® CTec 3)	Yeast extract, ammonium sulfate	46	144	180	14.8	1.24	[20]
ACCC20341	Tea waste	Chemical (sulfuric acid)	Yeast extract, ammonium sulfate	95.63	96	180	16.83	1.875	[15]
C23	Synthetic lignocellulosic-like medium	–	Yeast extract, ammonium sulfate	120	144	11	n.d	0.07	[16]
L/24–26-1	Sugarcane molasse	Chemical (sulfuric acid)	Yeast extract, ammonium sulfate	40	168	21.5	0.715	0.128	[17]
NCIM 3547	Neem oilseed cake	Chemical (microwave mild acid hydrolysis)	Yeast extract	25.83	96	60.88	0.024	0.88	[18]
DSM 4444	Spent coffee grounds	Biological (β-glucosidase Viscozyme)	Yeast extract	23.3	125	13	0.43	0.104	[19]
DSM 4444	**MTW**	Biological (novozymes NS22119)	None	9.1	30	89.9	32.69	2.9	***This study***
	MTB			15.7		163.5	26.37	5.5	
	SOW			7.7		123.8	41.96	4.1	
	SOB			13.8		362.7	163.37	12.1	
	eMTB			13.3		10.6	2.07	0.35	
	eSOB			12.1		25.27	6.01	0.84	

SOW, **SOB**, **MTW**, and **MTB**, as disclosed in the text, represent wood (**W**) and bark (**B**) of sessile oak (**SO**) and mulberry tree (**MT**). **eMTB** and **eSOB** represent the corresponding biomasses without the extractive components. n.d. = not determined

2.5 mg/L. Further data analysis shows that β-carotene is statistically ($p < 0.05$) more produced when the bark hydrolysate is used compared to the wood one, regardless of the biomass type, whereas this difference in torulene production can be observed only for mulberry tree, being bark hydrolysate superior to the wood hydrolysate ($p < 0.05$).

Calculation and comparison of carotenoid yields in the different conditions are summarized in Table 5, whereas Additional file 1: Fig. S1 permits to visualize the direct comparison between carotenoid's production profiles. These data are in accordance with previous findings disclosing that a C/N ratio of 80, as in **SOB** hydrolysate, is optimal to maximize β-carotene production over other carotenoids [63]. In addition, as already mentioned, the presence of gallates in **SOB** hydrolysate can be considered an additional triggering element for carotenogenesis [59].

Table 5 shows, however, that, considering the yield on hydrolysate sugars, **SOW** hydrolysate can be calculated to represent the best option, after 30 h of growth, for torulene production ($0.047 \pm 0.008\%$ g/g, $p < 0.05$), and the second-best option for β-carotene production ($1.61 \pm 0.20\%$ g/g). SOB hydrolysate remains the best choice ($p < 0.05$) considering β-carotene yields on both initial sugars in the medium and on consumed sugars (Table 5). **MT**-derived samples, in this calculation mode, fall behind the SO-derived ones, for both β-carotene and torulene production ($p < 0.05$).

This is consistent with previous data on *R. toruloides* growth on residual biomass hydrolysates, where the maximum production of carotenoids was reached between the end of the exponential phase and the entrance of the stationary one [34, 37]. In the case of **SOB**, the presence of linear growth rather than exponential one can be seen as indicative of the fact that the cells face hurdles, potentially due to the gallate presence. Indeed, compounds, such as phenols, interfering with the oxidative metabolism, or more generally stressful growth conditions, are known to act as a trigger to the production of carotenoids while being detrimental to growth [8, 64]. Therefore, when developing bioprocesses based on residual lignocellulosic biomasses, a trade-off between these actors has to be considered to maximize both growth and carotenogenesis.

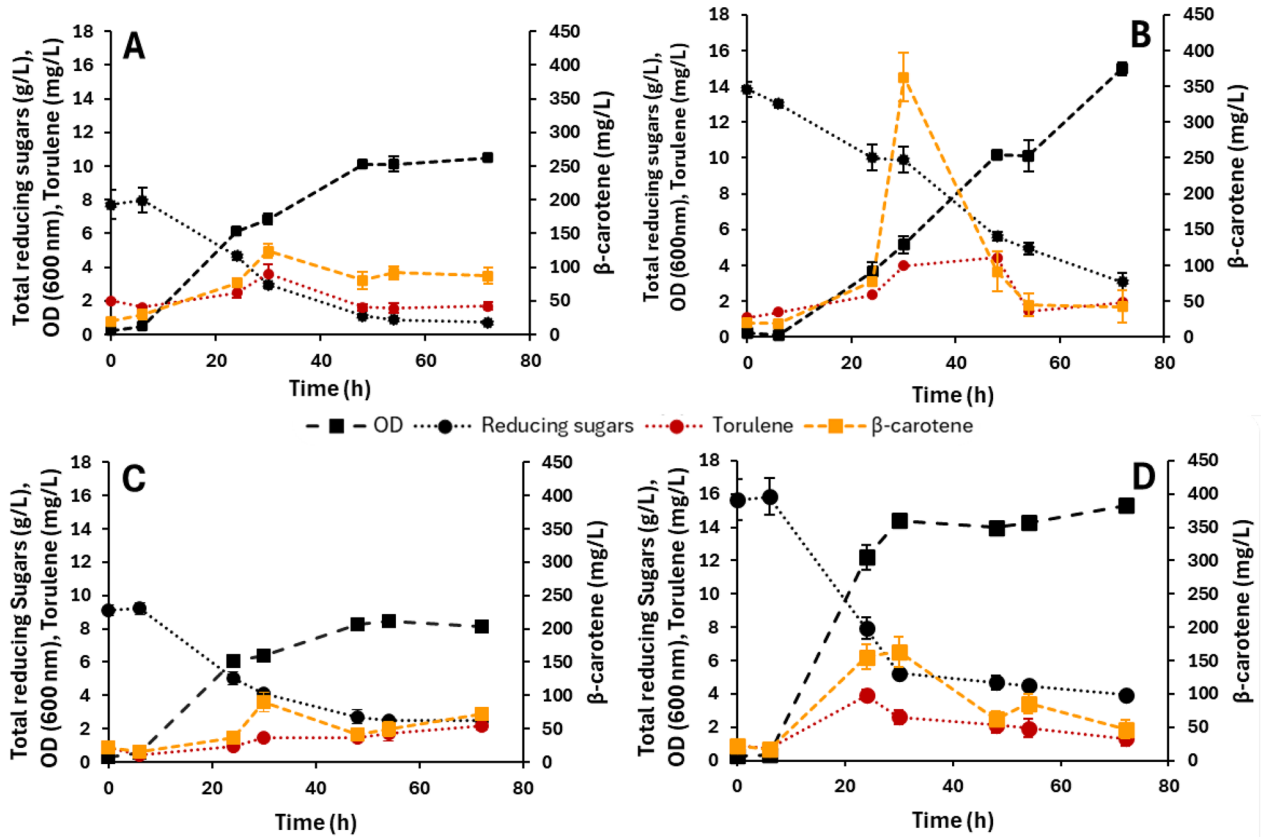

Fig. 4 Fermentation profile and carotenoids production by *R. toruloides* on **SOW** (**A**), **SOB** (**B**), **MTW** (**C**), and **MTB** (**D**) hydrolysates. Values are the means ± SD of three independent experiments

Table 5 Quantitative analysis and yields calculations of β-carotene and torulene production by *R. toruloides* from **SOW**, **SOB**, **MTW**, and **MTB** hydrolysates, after 30 h of fermentation

	Production (mg/L)		Yield on initial biomass (10^{-5} g/g)		Yield on hydrolysate sugars (g/g)		Yield on consumed sugars (g/g)	
	β-Carotene	Torulene	β-Carotene	Torulene	β-Carotene (%)	Torulene (10^{-2}) (%)	β-Carotene (%)	Torulene (10^{-2}) (%)
SOW	123.8 ± 10.6	3.6 ± 0.5	83 ± 7	2.4 ± 0.4	1.61 ± 0.20	4.7 ± 0.8	2.6 ± 0,4	7.6 ± 1.6
SOB	362.7 ± 33.8	4.0 ± 0.2	241 ± 23	2.7 ± 0.9	2.62 ± 0.25	2.9 ± 0.1	9,2 ± 1,1	10.1 ± 0.9
MTW	89.9 ± 14.8	1.5 ± 0.2	60 ± 10	1.0 ± 0.1	0.99 ± 0.16	1.6 ± 0.1	1.7 ± 0.3	2.9 ± 0.3
MTB	163.5 ± 22.6	2.6 ± 0.4	109 ± 15	1.7 ± 0.3	1.04 ± 0.16	1.7 ± 0.3	1.6 ± 0.3	2.5 ± 0.5

Values are the means ± SD of three independent experiments

To investigate whether also components comprised in the extractives could represent inhibitors, dedicated studies in this direction were performed. In fact, such compounds are typically present in woody biomasses like those valorised in this study while being limited or absent in other organic/lignocellulosic materials, such as wheat bran, tea, or molasses.

Effect of extractives on the production of carotenoids

To study the importance of extractives and/or impurities, **MTB** and **SOB** were chosen as biomasses, since the numbers and respective amounts of extractives in these bark samples were higher compared to the ones found for the wood samples ($p < 0.05$) (Table 1). Extractives were removed as described in "Removal of extractives

from grounded biomasses" section and extracted **MTB** and **SOB**, i.e., **eMTB** and **eSOB**, respectively, were subjected to enzymatic hydrolysis and SHF following the established procedures. Data obtained from growth in terms of optical density (OD), sugar concentration over time, and carotenoids produced allowed for determining the impact of the presence or absence of extractives in the biomasses. Data are summarized in Fig. 5.

Figure 5 shows that the elimination of extractives did not have an impact on enzymatic hydrolysis, since the amount of released sugars in the starting medium is comparable regardless of their presence. The elimination of extractives was expected to improve microbial performance and, in turn, the production of carotenoids. Indeed, **eSOB** hydrolysate permits for a higher OD compared to **SOB**, despite a comparable sugar consumption, suggesting that the extractives are responsible for impairing yeast growth. On the other hand, no significant difference can be observed between growth on **MTB** and **eMTB**. Surprisingly and independently from growth recovery, the absence of extractives in **eSOB** and **eMTB** resulted in a reduced accumulation of carotenoids (Fig. 5C, D), compared to **SOB** and **MTB** derived carotenoids ($p < 0.05$), implying that the presence of extractives, while being inhibitors, acts as a trigger for carotenogenesis. In fact, the production of carotenoids on **eMTB** and **eSOB** hydrolysates is comparable to previous studies whose starting feedstocks for *R. toruloides* do not typically contain a significant amount of such extractives, e.g., wheat bran, tea, molasses (Table 4). Indeed, some of the molecules identified in either **SOB** or **MTB** extractives (Additional file 1: Table S1) are associated with antimicrobial activity, like in the case of lupeol [65], β-amyrin [66] and benzaldehyde [67] possibly causing a stressful environment leading to carotenogenesis. These observations provide novelty in terms of understanding that extractive components of lignocellulosic biomasses can act as promoting elements for the production of the desired molecule, especially when it is triggered by stressful conditions like in carotenogenesis. These findings are in line with previous literature, which suggest the role of different types of stress in the production of carotenoids by *R. toruloides* [8]. Furthermore, in terms of biorefinery development, detoxification of the initial biomass is not needed in this case, making the process simpler.

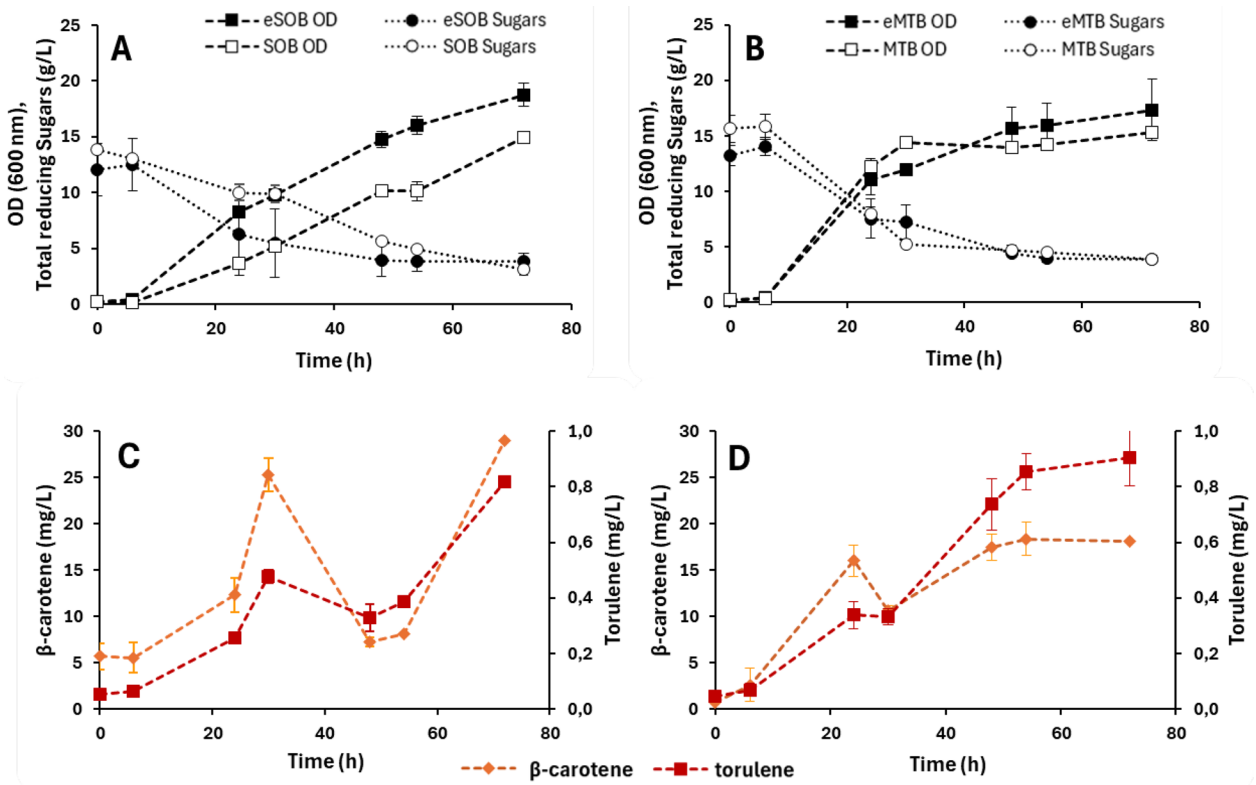

Fig. 5 Fermentation profiles of *R. toruloides* during growth on **eSOB** (**A**) and **eMTB** (**B**) hydrolysate. In these panels, data regarding the fermentation on **SOB** or **MTB** hydrolysate are repeated from Fig. 4 to highlight differences. Production of carotenoids from **eSOB** (**C**) and **eMTB** (**D**). Values are the means ± SD of three independent experiments

These data also suggest that growth and production of carotenoids are not strictly correlated, depending on the environmental conditions. Microbial nutritional needs and biomass composition can thus be correlated to the production of the desired compounds, which is a relevant aspect in the field of bioprocesses and biorefineries. Further investigations, beyond the scope of this work, might involve genomic and transcriptomic analysis of carotenogenesis-related genes in *R. toruloides*, to better understand the effect of extractives, both as cohorts or as single compounds, on cellular behavior and regulation.

Conclusion

This study demonstrates the potential of leveraging diverse urban lignocellulosic residues as feedstock for microbial-based biorefineries. The findings here disclosed confirm that the enzymatic hydrolysis effectively releases fermentable sugars from previously underutilized sessile oak and mulberry tree biomasses. *R. toruloides* exhibited robust performance, growing across all hydrolysates regardless of their sources, and efficiently accumulating carotenoids, specifically β-carotene and torulene. The observation that natural inhibitors present in the hydrolysates stimulate carotenoid biosynthesis underscores a critical element: challenging biomass components can, in fact, enhance the formation of desired products, potentially optimizing bioprocess without requiring intensive pre-treatment for inhibitor removal. Interestingly, when extractive components were removed, carotenoids production decreased to levels comparable with previous studies using other biomasses.

By transforming local, readily available waste streams into valuable bioproducts, this research provides a practical example of a robust valorisation of biodiversity and woody bio-waste. Specifically, this biorefinery approach successfully valorises regional woody biomass for the production of high-value pigments and food additives, broadening the scope of biodiversity valorisation. Future works will focus on further optimizing the bioprocess and deepening the understanding of the interactions between specific inhibitors and carotenogenesis. Furthermore, given the intrinsic differences between hardwood and softwood in terms of material density and polymer composition which both can affect enzymatic efficiency, investigating the effect of different tissues of the same plant on the process remains a valuable area to explore. Finally, to truly "close the loop" in this biorefinery concept, investigations into the valorisation of the remaining lignin-rich residues are paramount.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13068-026-02749-3.

Additional file 1. **Table S1**: Composition of wood and bark samples, used in this study, determined by GC/MS analyses. Compounds of each class are listed in ascending order of retention time. **Figure S1**: Development of (A) β-carotene and (B) torulene production by *R. toruloides* from SOW, SOB, MTW, and MTB hydrolysates. Values are the means ± SD of three independent experiments.

Author contributions

S.B., F.S., G.M.B., M.D., and V.T. were involved in investigation and data curation. S.B., F.S., V.T., H.L., and P.B. were involved in conceptualization. S.B. was involved in writing—original draft preparation. S.B., F.S., V.T., D.P., M.O., H.L., and P.B. were involved in writing—reviewing and editing. D.P., M.O., H.L., and P.B. were involved in funding acquisition and supervision.

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Data availability

No data sets were generated or analyzed during the current study.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

S.B. declares to be part of the editorial board of Biotechnology for Biofuels and Bioproducts. The authors declare they do not have any further competing interests as defined by BMC or other interests that might be perceived to influence the results and/or discussion reported in this paper.

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