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An experimental cascade biorefinery from orange residues: Sequential recovery of bioactive compounds, pectin, and fermentation of sugar-rich side streams using conventional and non-conventional yeasts

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ABSTRACT

The valorization of fruit-derived residues under the biorefinery concept has been a topic of interest in the last years due to the presence of high value-added substances in their composition. However, feasible alternatives for their implementation at an industrial level are still being developed since the abundance of pectin and extractives has made its biorefining challenging compared to conventional lignocellulosic residues. In this study, the sequential valorization of Orange Residues (OR) in a biorefinery was evaluated following the principles of biomass cascading and considering the composition of residual streams as a valuable input to maximize recovery after each processing step, without focusing on a sole product. To extract full value from the side-streams, fermentation with conventional and non-conventional yeasts was explored. The proposed biorefinery sequence produced essential oils, phenolic compounds, pectin, and fermentable sugars that were later converted to ethanol, xylitol, and single-cell protein. A detailed mass balance allowed to track compositional changes throughout the cascade and identify how extraction substances accumulate after each step, affecting further processing and side-stream utilization. The sequence proposed in this work extracted/transformed ~85 % of the initial biomass into value-added products.

1. Introduction

In the last few years, the number of biorefinery projects and installed biorefineries has been growing. A report made in 2017 states that in Europe, at least 224 biorefineries were operating, most of them dedicated to transforming oils and fats through oleo-chemistry and sugar or starch to ethanol and other chemicals [1]. More detailed research shows that in 2018, the European Union had 803 biorefineries, most of which produce bio-based chemicals (507), and the rest liquid biofuels (363) and bio-based composites and fibers (141) [2]; the reported biorefineries are mainly located in The Netherlands and Belgium, using primordially agricultural waste. In other parts of the world, the implementation of cellulosic bioethanol biorefineries (including other byproducts like starch, animal feed, or CO_2) in the United States is well-known, and Australia has the potential to support large-scale biorefineries [3]. Most of the existent biorefineries have in common the use of lignocellulosic feedstocks with a similar composition, such as wood, sorghum, rice husk, corn stover, and sugarcane bagasse, with challenges far less significant than the processes that must handle variable composition [4].

In contrast, biorefineries derived from fruit residues have been mostly limited to case studies that have been evaluated in the literature [5–7]. The feasibility of using these biomasses presents novel opportunities for the extraction of value-added products but their valorization depends highly on composition [8]. Fruit-derived biomasses contain high amounts of extractives and pectin, which have high value but restrict the way the biomass is processed in a biorefinery [9]. In contrast, their low lignin content eliminates the need for pretreatments [10]. It is known that galacturonic acid, the main constituent of pectin, could be difficult to recover from these residues since it could be easily degraded

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Table 1
Examples of biorefineries from Orange Residues (OR) reported in the literature.

1st step	2nd step	3rd step	4th step	5th step	Details	Reference
Enzymatic extraction of essential oils and pectin	Simultaneous saccharification and fermentation to produce ethanol	–	–	–	- The process is targeted to ethanol production. - The content of extractives is not reported. - Valuable compounds could be degraded during essential oil recovery (boiling).	[16]
Sulfuric acid hydrolysis to produce pectin	Anaerobic digestion of the solid residue	–	–	–	- The process is targeted to the hydrolysis step. - The content of extractives and protein is not measured. - Valuable compounds could be degraded during hydrolysis.	[17]
Enzymatic hydrolysis and fermentation with *S. cerevisiae* for ethanol production	Anaerobic digestion of the solid residue	–	–	–	- The process is targeted to biogas production. - The content of pectin, extractives, and protein is not measured.	[18]
Ensiling for biomass storage and production of organic acids and ethanol	Pectin production or animal feed	Anaerobic digestion of the solid residue	–	–	- The process is targeted to ensiling. - Ensiling could cause the loss or degradation of valuable compounds. - The composition of the biomass is not reported.	[19]
Biodegradation of biomass with white-rot fungi	Anaerobic digestion of the solid residue	–	–	–	- The process is targeted to biogas production. - Biodegradation removes terpenes and phenolics but causes the degradation of valuable compounds. - Potential use of fungal biomass as a food source is not considered. - The content of pectin is not reported.	[20]
Acid hydrolysis with HCl or H_3PO_4 to produce pectin	Carbonization of the solid residue to produce activated carbon	–	–	–	- The process is targeted to pectin production. - Valuable compounds could be degraded during hydrolysis and carbonization. - Carbonization could cause the release of harmful substances. - The composition of the biomass is not fully described.	[21]
Hydrothermal treatment to release fermentable liquor	Enzymatic hydrolysis of the solid residue	Production of Biobutanol, biohydrogen, and organic acids through fermentation.	Anaerobic digestion of the solid residue	–	- The process is targeted to fermentation. - The content of extractives is not reported. - Valuable compounds could be degraded during hydrothermal treatment.	[22]
Aqueous extraction of free sugars	Extraction of essential oils with steam distillation	Extraction of phenolic compounds with ethanol	Pectin production through citric acid hydrolysis	Production of sugars through hydrolysis (sulfuric acid and enzymes) fermentation of bacterial cellulose	- The process is not targeted into a specific product. - Manual peeling of the flavedo was required for essential oils extraction. - Processing conditions could be optimized. - The composition of the biomass is fully described.	[23]
Sulfuric acid hydrolysis to release compounds	Recovery of essential oils with expansion tanks	Fermentation of hydrolysate to produce ethanol with *S. cerevisiae*	Anaerobic digestion of the solid residue		- The process uses literature data for simulation and is focused on the hydrolysis step. - Valuable compounds could be degraded during hydrolysis.	[24]

(*continued on next page*)

Table 1 (*continued*)

1st step	2nd step	3rd step	4th step	5th step	Details	Reference
					- The composition of the biomass is not fully described. - The content of extractives, protein, and ash is not reported.	
Microwave Hydrodiffusion and Gravity or by steam distillation of essential oils	Ultrasound-assisted extraction or conventional solvent extraction of phenolic compounds	Microwave assisted extraction or conventional extraction of pectin with HNO_3	–	–	- The process is targeted to the recovery of phenolic compounds and pectin using specialized equipment. - The composition of the biomass is not fully described. - Valuable compounds could be degraded during microwave extractions.	[25]
Sulfuric acid hydrolysis to release compounds	Recovery of essential oils with expansion tanks	Production of pectin	Fermentation of remaining sugars to ethanol	Anaerobic digestion of the residual streams	- The process is targeted to the hydrolysis step. - The composition of the biomass is fully described. - Valuable compounds could be degraded during hydrolysis.	[26]
Hydrodistillation of essential oils	Sulfuric acid hydrolysis to produce pectin	Fermentation of remaining sugars to succinic acid	Production of fertilizer with the solid residue	–	- The process is targeted to fermentation. - The content of pectin, extractives, protein, and ash is not measured. - Valuable compounds could be degraded during hydrolysis.	[27]
Microwave treatment to release compounds	Recovery of pectin by precipitation	Recovery of bio-oil with liquid-liquid extraction	Cellulose is recovered as the solid residue	–	- The process is targeted to microwave treatment. - The composition of the biomass is not fully described. - Valuable compounds could be degraded during microwave treatment.	[28]
Steam distillation of essential oils	Pectin production through citric acid hydrolysis	Anaerobic digestion of the solid residue	–	–	- The process is targeted to the recovery of essential oils and pectin. - Processing conditions could be optimized. - The composition of the biomass is fully described. - Other valuable compounds, like fibers and phenolic compounds, are not valorized.	[29]
Pectin production through citric acid hydrolysis	Fermentation of remaining sugars to mucic acid	Anaerobic digestion of the solid residue	–	–	- The process is targeted to the recovery of pectin and mucic acid. - Processing conditions could be optimized. - The composition of the biomass is fully described. - Other valuable compounds, like essential oils, fibers, and phenolic compounds, are not valorized.	[30]
Hydrodistillation of essential oils	Sulfuric acid hydrolysis to produce pectin	Enzymatic hydrolysis of the solid residue	Fermentation of hydrolysate to produce ethanol	Anaerobic digestion of the residual streams	- The composition of the biomass is fully described. - Processing conditions could be optimized. - The process is not targeted into a specific product. - Valuable compounds could be degraded during hydrolysis.	[31]
Microwave hydrodistillation of essential oils	Pectin production from residual liquid fraction	–	–	–	- The process is targeted to microwave treatment. - The composition of the biomass is not fully described.	[32]

(*continued on next page*)

Table 1 (*continued*)

1st step	2nd step	3rd step	4th step	5th step	Details	Reference
Solid-liquid extraction of essential oils with hexane and ethanol	Dark fermentation to produce volatile fatty acids	Anaerobic digestion of the solid residue	–	–	- Valuable compounds could be degraded during microwave treatment. - The process is targeted to biogas production. - The composition of the biomass is not fully described. - The use hexane could represent safety issues. - Other valuable compounds, like pectin, fibers, and phenolic compounds, are not valorized.	[33]
Fractionation of biomass using subcritical water	Recovery of sugars and flavanones	–	–	–	- The process is targeted to the use of subcritical water. - The composition of the biomass is fully described. - Valuable compounds could be degraded during hydrothermal treatment. - Other valuable compounds, like pectin and essential oils are not valorized.	[34]
Solar hydrodistillation of essential oils	Pectin production from residual liquid fraction	Extraction of phenolic compounds	–	–	- The process is targeted to the use of solar hydrodistillation. - The composition of the biomass is not fully described. - Valuable compounds could be degraded during hydrodistillation.	[35]

at high temperatures [10,11]. Also, certain essential oils and polyphenols could interfere with other processes, like fermentation [12,13]. It is clear that the valorization of fruit residues in a biorefinery requires, as a critical design step, considering their whole composition and its effect in the upgrading techniques that could be applied [14].

Orange Residues (OR), obtained from juice processing, are one of the most abundant fruit-derived biomasses generated worldwide [8,15]. Different configurations of biorefineries from OR have been evaluated in the literature with the aim of proposing feasible alternatives for their implementation (Table 1). Even though most of the available examples intend to follow the biorefinery concept, most of them usually focus on a sole substance/step or use techniques that alter the characteristics of the residual biomass. For the integral valorization of OR in a biorefinery it is necessary to use combined processes that aim to valorize all biomass components and avoid leaving behind or cause the degradation of valuable substances by carefully linking different valorization methods [8].

Consequently, this work evaluated a OR biorefinery that follows the principles of biomass cascading by considering the compositional changes of biomass along the process. In that way, high-value substances were carefully recovered in each step from the biomass and all side streams were used, which maximized the overall recovery of substances in the cascade. A biorefinery sequence, not focused on specific components or steps, was evaluated based on previous results that compared different valorization techniques of OR and their operating conditions. The cascade use of steam distillation, solid-liquid extraction, acid hydrolysis, and the fermentation of side-streams with conventional and non-conventional yeasts allowed to identify yields, compositional changes, processing alternatives, and challenges that need to be addressed for the feasible integral implementation of a biorefinery from OR.

2. Materials and methods

2.1. Sample collection and preparation

Orange residues (OR) (i.e., peels, seeds, and remnant Pulp) were collected at an orange juice shop at Universidad de Los Andes. Residues were milled (<5 mm) (Food Processor WRP-FP-408, Würden) and dried at 45 °C in a convection oven (FD 115, Binder) to a moisture content <10 % (Moisture analyser MTB 64M, VWR) and then used for processing. For compositional analysis, samples were milled (<1 mm) in a universal cutting mill (Pulverisette 19, Fritsch), following the NREL procedures [36]. The composition of the OR, consisted of 55.78 ± 0.71 % of extractives, 22.36 ± 0.84 % of pectin, 7.18 ± 0.52 % of cellulose, 5.73 ± 0.53 of hemicellulose, 4.31 ± 0.05 % of protein, 3.94 ± 0.22 % of lignin, 3.33 ± 0.04 of ash, and 1.48 ± 0.01 % of starch.

2.2. Evaluation of a biorefinery for the sequential valorization of orange residues

The biorefinery followed the sequence proposed by the authors at the optimal process conditions that were found in previous work [37]. OR were first submitted to Steam Distillation (SD) to extract essential oils, then to Solid-Liquid Extraction (SLE) with a water-ethanol solution to recover free sugars and phenolic compounds, after that hydrolyzed with citric acid to recover pectin, and finally hydrolyzed with sulfuric acid to recover fermentable sugars, as shown in Fig. 1.

The process began by loading 200 g of previously prepared OR to a steam distillatory system (Laboratory Scale Essential Oil Extractor, Figmay) in which water was heated at 1500W and kept at a constant level of ~2L. The steam produced flowed through the sample to perform the extraction [29]. The essential oils were recovered after condensation, decanted using the collector system that comes with the equipment, and finally the yield was determined gravimetrically. The solid residue after SD was used for SLE by placing it in Schott flasks (2L) using

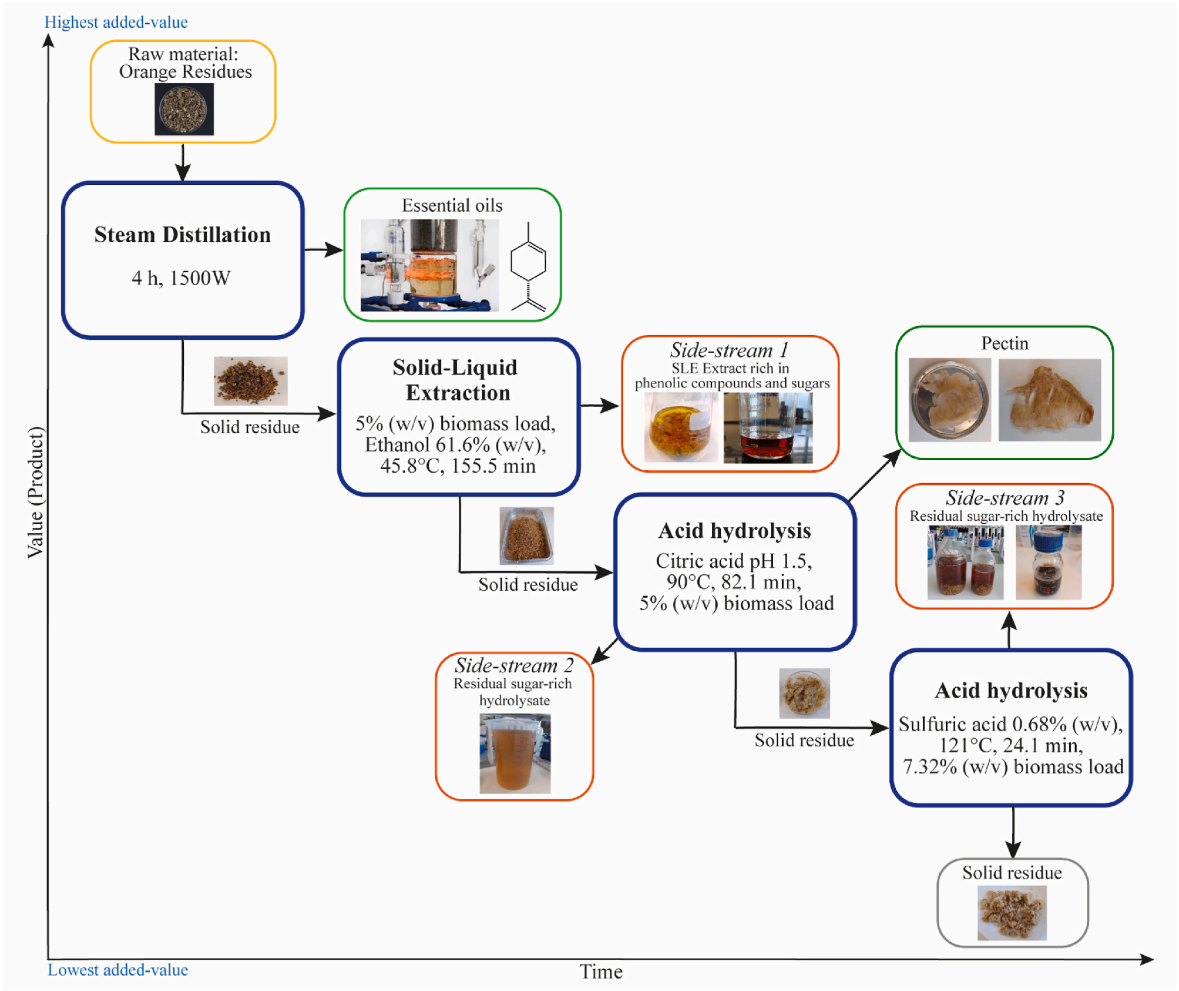

Fig. 1. Scheme of the biorefinery cascade from Orange Residues (OR). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

biomass load of 5 % (w/v) and mixing it with 61.6 % (w/v) ethanol. The flasks were then placed in a Shaker (Incubator Shaker Innova 42, New Brunswick) kept at 100 rpm and 45.75 °C for 155 min (~2.6 h). The SLE extract (Stream 1) was recovered with help of a cheesecloth and stored at −20 °C for further analysis and use. The solid residue from SLE was then used for pectin production by loading the sample in Schott flasks (2L) using a biomass load of 5 % (w/v) and mixing it with a citric acid solution (pH 1.5). The samples were placed in a hot water bath (Heating Thermostat Alpha A6, Lauda) at 90 °C for 82 min, after which the samples were centrifuged for 15min at 8000 rpm (Heraeus Multifuge X3R, Thermo Scientific) and filtered with a cheesecloth. The recovered hydrolysate was then mixed with 96 % ethanol in a ratio of 2:1 (v/v) for 18 h at 6 °C to precipitate pectin [38]. Pectin was then recovered using a spatula, centrifuged for 15min at 8000 rpm (Heraeus Multifuge X3R, Thermo Scientific), filtered with a cheesecloth, further washed with absolute ethanol, and dried in a convection oven at 40 °C for 24 h [21]. The residual hydrolysate recovered after pectin precipitation (Stream 2) was recovered and stored at −20 °C for further analysis and use. Finally, the solid residue from pectin extraction was used for fermentable sugar production by placing it in Schott flasks (2L) where sulfuric acid 0.68 % (m/v) was mixed at a 7.32 % (m/v) of biomass load. The hydrolysis was carried out in an autoclave (Benchtop Autoclave CertoClav Multicontrol, CertoClav) at 121 °C for 24.1 min. The mixture was centrifuged for 15min at 8000 rpm and filtered using a cheesecloth to recover the sulfuric acid hydrolysate (Stream 3) which was stored at −20 °C for further analysis and use. After each processing step of the biorefinery, a sample from the solid residue was recovered for compositional analysis. Aliquots from the liquid fractions were also recovered to determine their content of sugars, organic acids, furans, phenolic compounds, and protein.

2.3. Compositional analysis of the residual solid biomass obtained after each processing step

Chemical composition was determined for the OR and the samples recovered after each processing step according to the NREL protocols. Ash content was determined using a muffle ramp up to 575 °C (Type F62700 Furnace, Barnstead International) [39]. Protein content with the Kjeldahl method [40] using a Nitrogen to protein conversion factor of 6.25 [41]. For extractives, Soxhlet extractions were performed sequentially with water, ethanol, and hexane [42] and measured gravimetrically after solvent removal with the help of a rotary evaporator (RV 10 digital V Rotary evaporator, IKA). Extractive-free samples were used for structural carbohydrates and lignin determination through acid hydrolysis according to the NREL protocols [43]. Starch was determined from non-cellulosic glucan [44] and pectin was measured using chemical and enzymatic treatment to release galacturonic acid from the extractive-free samples which was measured by HPLC [45]. The proximate analysis, moisture content, total solids, volatile matter, and ash were determined according to the ASTM methods E17656, 1755, and E872. The ultimate analysis (i.e., contents of C, N, H, S, and O by difference) was determined according to the ASTM D5373-16 method and

the calorific value was determined according to the ASTM E711-06 method.

2.4. Mass balance of the cascade biorefinery

A global mass balance was performed considering a calculation basis of 100 g of OR and using the yields, compositions, compound concentrations (g/L), and volumes obtained from the biorefinery sequence. The composition of the residual solids from each step was normalized by adjusting the totals to 100 % in all cases. The data obtained before and after each processing step was used to find the value in mass (g) of each substance involved.

2.5. Fermentation of side-streams using conventional and non-conventional yeasts

2.5.1. Microorganism, inoculum, and cultivation conditions

The side-streams obtained from the biorefinery described in 2.2 were used for fermentation to produce ethanol, xylitol, and single-cell protein. A commercial strain of *Saccharomyces cerevisiae* Ethanol Red was used for ethanol production at 30 °C. An evolved strain of the thermotolerant *Kluyveromyces marxianus* NRRL Y-6373 obtained by adaptive laboratory evolution (at BCBT group, DTU, unpublished data) was used for ethanol and xylitol production at 40 °C. The methylotrophic yeast *Hansenula polymorpha* CBS 4732 was used for the production of single-cell protein and ethanol. These yeasts have shown promising results for the conversion of C5 and C6 hydrolysates [46–49]. The strains were provided by the strains collection of *The Biomass Conversion and Bioprocess Technology (BCBT) group* from *The Technical University of Denmark*.

Stock cultures were maintained at − 80 °C with 20 % glycerol in Yeast Extract–Peptone–Dextrose (YPD) medium composed of (g/L): Glucose, 20; peptone; 20; and yeast extract, 10. For cell activation (pre-inoculum), 1 cryovial of each microorganism was unfrozen and activated in 50 mL of YPD for 18h. The culture conditions were 30 °C and 250 rpm for *S. cerevisiae*, 40 °C and 150 rpm for *K. marxianus*, and 37 °C and 250 rpm for *H. polymorpha*, according to previous studies [46,49].

Afterward, an aliquot of 1 mL of activated cells was transferred to the inoculum media for cell propagation at the same conditions of the activation stage. The inoculum media was formulated at the same sugar concentration (glucose, fructose, xylose, arabinose) of the side-streams. It also contained as nutrients: 3 g/L yeast extract, 5 g/L $(NH_4)_2HPO_4$, 3 g/L KH_2PO_4, 0.24 g/L $MgSO_4{\cdot}7H_2O$, and 2.54 g/L microminerals (15 mg/L EDTA, 4.50 mg/L $ZnSO_4{\cdot}7H_2O$, 0.30 mg/L $CoCl_2{\cdot}4H_2O$, 0.84 mg/L $MnCl_2$ mg/L·4H2O, 0.30 mg/L $CuSO_4{\cdot}5H_2O$, 3 mg/L $FeSO_4{\cdot}7H_2O$, 0.40 mg/L $NaMoO_4{\cdot}$2H2O, 1 mg/L H_3BO_3, and 0.1 mg/L KI) [48].

After 18 h of cultivation, the cells were centrifuged (Heraeus Multifuge X3R, Thermo Scientific) at 8000 rpm, 10 min, and 4 °C, washed twice with saline solution (NaCl 0.9 % (w/v)), and inoculated in the fermentation media (further described in 2.4.3). Polypropylene square 24-deepwell microplates (microplates CR1424, covers CR1224a, and clamps CR1700, EnzyScreen) were used for the fermentation experiments at the same conditions of the activation stage. The assays were performed in duplicate using a working volume of 1.5 mL. The pH of the media was adjusted to 5.8 and sterilized with a 0.22 μm polyethersulfone membrane, in all cases [48].

2.5.2. Preparation of biorefinery side-streams for fermentation

The SLE extract (Stream 1) and the hydrolysates (Stream 2 and Stream 3) from the biorefinery were filtered with a 0.45 μm polyethersulfone membrane to remove humins [46]. Afterward, a treatment was applied to remove citric acid and acetic acid from the hydrolysates by first neutralizing them with ~75 mL of NaOH 10 % (w/w) until a pH between 8 and 9 was reached (forming trisodium citrate and sodium acetate) and then dissolving 375 mL of $CaCl_2$ 28.5 % (w/w) (forming $Ca(OH)_2$) [50,51]. The mixture was continuously stirred and heated with the help of a magnetic stirrer (RCT Basic, IKA) until boiling (~97 °C) to cause the precipitation of calcium citrate and calcium acetate [52,53]. Upon precipitation the mixture is removed from the heat and vacuum filtered with a 0.45 μm polyethersulfone membrane. The precipitate was further washed three times with hot water during filtering to recover remnant sugars and purify the salts [54]. The recovered liquid was then let to cool to room temperature and filtered again to remove any additional precipitation. After that, a rotary evaporator (RV 10 digital V Rotary evaporator, IKA) operating at 40 °C, 60 rpm, and 100 mbar, was used to partially remove ethanol and concentrate the samples.

2.5.3. Bioconversion of biorefinery side-streams into value-added products

For each yeast, the fermentation of the biorefinery side-streams was evaluated using a Two-way Anova experimental design to determine the effect of the fermentation media and the presence of nutrients in the production of value-added substances. Three side-streams obtained from the biorefinery from orange residues (Fig. 1) will be used as fermentation media: (i) The SLE extract, (ii) The residual hydrolysate recovered from citric acid hydrolysis after pectin extraction, and (iii) The hydrolysate obtained from the sulfuric acid hydrolysis. These fermentation media were evaluated with and without the addition of the nutrients listed in 2.4.1. After preparing the fermentation media, the initial sugar concentration (Sum of C6 and C5 sugars) was adjusted to 40 g/L with the help of a rotary evaporator (RV 10 digital V Rotary evaporator, IKA) operating at 40 °C, 60 rpm, and 100 mbar. A control experiment was included simulating the sugar content present in the hydrolysates without inhibitors. During the fermentations samples were taken to monitor cell growth by registering the optical density (OD) at 600 nm with the help of a portable cell density meter (Ultrospec 10 Cell Density Meter, Biochrom). These samples were then centrifuged to recover the supernatant for further measurement of pH (FiveEasy Plus pH meter FP20 with a pH sensor LE422, Mettler Toledo) and HPLC analysis. Besides, cell morphology was observed using an inverted microscope (Eclipse Ti2 Fluorescence microscope, Nikon).

2.5.4. Investigation of inhibitor's effect using simulated media (synthetic media)

The effect of inhibitors (acetic acid, citric acid, and ethanol) in the yeasts was investigated by using synthetic media simulating the same concentrations of sugars and inhibitors measured in the side-streams. Fermentations were carried out under the same conditions and using the procedures described in section 2.4.3.

2.5.5. Determination of Minimum Inhibitory Concentration (MIC)

The growth profile of the yeasts was evaluated at different concentrations of citric acid and ethanol to determine the Minimum Inhibitory Concentration (MIC) [55,56], or the "lowest concentration of an antimicrobial agent that inhibits the growth of a microorganism" [57]. Cultures of each yeast were performed in 96 well-microtiter plates [58] at the same conditions used in the fermentations and same sugar compositions of the side-streams but including two-fold dilution series of citric acid and ethanol starting from to 50 g/L and down to 6.25 g/L [57]. Cells were diluted to OD_{600} of 0.1 and grown for 24 h in triplicate for each strain. Cell growth was monitored by registering the OD_{600} nm every 30 min with the help of a microplate reader (SpectraMax iD3 Multi-Mode Microplate Reader, Molecular Devices).

2.6. Analytical methods

2.6.1. UHPLC quantification of sugars, organic acids, furans, ethanol, glycerol, and xylitol

Samples were measured using an Ultra-High-Performance Liquid Chromatographer (UHPLC Dionex UltiMate 3000, Thermo Fisher) equipped with a binary pump, online degasser, autosampler, a thermostated column compartment, a Shodex RI-101 Refractive Index Detector (RID), and a Diode Array Detector (DAD) used for the detection

Table 2
Chemical composition of side streams before (raw) and after preparation treatments.

Substance (g/L)	Stream 1		Stream 2			Stream 3	
	Raw extract	After treatment	Raw extract	After pectin recovery	After treatment	Raw extract	After treatment
Cellobiose	1.56 ± 0.05	0.61 ± 0.20	0.85 ± 0.05	0.073 ± 0.002	0.57 ± 0.01	0.73 ± 0.18	5.91 ± 0.57
Glucose	6.74 ± 0.06	26.46 ± 1.36	0.74 ± 0.19	0.42 ± 0.03	10.10 ± 0.44	1.01 ± 0.08	4.98 ± 0.78
Fructose	5.81 ± 0.1	26.85 ± 0.85	N. D.	N. D.	N. D.	N. D.	N. D.
Xylose	N. D.	N. D.	0.86 ± 0.14	0.345 ± 0.10	16.42 ± 0.83	1.98 ± 0.10	19.91 ± 1.95
Arabinose	N. D.	N. D.	0.79 ± 0.12	0.20 ± 0.03	1.98 ± 0.14	1.39 ± 0.06	20.43 ± 1.97
Citric acid	1.21 ± 0.06	2.14 ± 0.16	48.37 ± 6.7	19.23 ± 0.20	181.21 ± 0.20	30.63 ± 0.63	60.29 ± 1.50
Acetic acid	N. D.	N. D.	3.83 ± 0.07	1.34 ± 0.02	2.75 ± 0.14	1.80 ± 0.01	8.00 ± 0.72
HMF	N. D.	N. D.	0.02 ± 0.01	0.017 ± 0.01	0.04 ± 0.002	0.055 ± 0.13	0.23 ± 0.03
Furfural	N. D.	N. D.	N. D.	N. D.	N. D	N. D.	N. D.
Ethanol	450.2 ± 0.52	89.39 ± 1.74	450.2 ± 0.52	347.4 ± 0.78	N. D.	209 ± 0.10	75.64 ± 0.73

Stream 1: Solid-liquid extract, Stream 2: Citric acid hydrolysate, Stream 3: Sulfuric acid hydrolysate, N. D.: Not detected.

furans (254 nm). For compositional analysis, the content of sugars (Glucose, xylose, arabinose, mannose, and galactose) was measured using a Biorad Aminex HPX-87P column (300 × 7.8 mm, 9 μm particle size, and 8 % cross-linkage) (Bio-Rad, USA) operated at 85 °C, with HPLC grade water as the mobile phase, a flow rate of 0.6 mL/min, an injection volume of 20 μL, and a run time of 25 min [43]. For the extracts, hydrolysates, and fermentations, the quantification of sugars (Glucose, fructose, xylose, arabinose), organic acids (citric acid, acetic acid, lactic acid, and galacturonic acid), ethanol, glycerol, and xylitol, was measured using a Biorad Aminex HPX-87 H column (1300 × 7.8 mm, 9 μm particle size, and 8 % cross-linkage) (Bio-Rad, USA) operated at 60 °C, with 0.005 M Sulfuric acid as the mobile phase, a flow rate of 0.6 mL/min, an injection volume of 20 μL, and a run time of 30 min [46]. Calibration curves were constructed at 0.025–5 mg/mL for sugars, organic acids, ethanol, glycerol, and xylitol, and at 0.025–0.5 mg/L for furfural and 5-HMF (Fig. S1). The assays were performed in triplicate.

2.6.2. Total phenolic content (TPC)

TPC was determined using the microscale version of the Folin-Ciocalteu method [59]. A Gallic acid calibration curve between 5 and 500 mg/L was used, and results were expressed as gallic acid equivalents (GAE). Samples of 20 μL were mixed with 1.58 mL of distilled water and 100 μL of the Folin-Ciocalteuś (Sigma-Aldrich) reactive (2N). After mixing the samples for 8 min, 300 μL of sodium carbonate at 20 % (w/v) (PanReac-AppliChem ITW) was added. Samples were incubated at room temperature for 2 h in the dark. Absorbance was measured using a microplate reader (SpectraMax iD3 Multi-Mode Microplate Reader, Molecular Devices) for UV–Vis detection at 765 nm. Distilled water was used as the blank.

2.6.3. Protein content

Quantification of the protein content in the hydrolysates was performed using the ninhydrin assay [60]. This method allows the colorimetric measurement of protein without the interference of sugars and ethanol in absorbance. A Bovine Serum Albumin calibration curve between 20 and 200 μg/mL was used. Samples (40 μl) were transferred into Eppendorf tubes and mixed with NaOH 13.5 M (60 μl) of NaOH to perform alkaline hydrolysis at 121 °C for 20 min in an autoclave (Benchtop Autoclave CertoClav Multicontrol, CertoClav). After that, each sample was neutralized with 100 μl of glacial acetic acid. The colorimetric reaction was done by adding 200 μl of Ninhydrin 2 % (w/v) and incubating the samples for 20 min at 100 °C in a heating block (Eppendorf ThermoMixer C, Eppendorf). The reaction was stopped with the help of an ice bath and adding 1 mL of ethanol 50 % (v/v). Absorbance was measured using a microplate reader (SpectraMax iD3 Multi-Mode Microplate Reader, Molecular Devices) for UV–Vis detection at 570 nm. Distilled water was used as the blank.

2.7. Statistical analysis

Data were analyzed using the software Statistica 12® for data analysis and visualization. The significance level of the differences between means was determined using a Tukey test ($p < 0.05$). Related assumptions were carefully validated, such as equal variances (Bartlett's Test) and normal distribution (Anderson-Darling test).

3. Results and discussion

3.1. Yields of extraction in the biorefinery

From the initial 200 g of OR, a yield of 0.70 ± 0.037 g/100 g DM of essential oils (97 % D-Limonene and 3 % of α-Myrcene) were obtained from SD. The solid residue from SD (~196.6 g) was used for SLE with water and ethanol, from which 42.46 ± 0.85 g/100 g DM of an extract rich in sugars and phenolic compounds were obtained. For both SD and SLE, the yields obtained were similar to those obtained in previous work [37]. After that, the extract-free solid (~106 g) was hydrolyzed with citric acid resulting in 24.26 ± 0.29 g/100 g DM of pectin and a sugar-rich liquid fraction that was recovered for further use. The yield of pectin was higher than the one found previously at the optimal conditions by the authors [37]. Free sugar removal during SLE could have been beneficial for the hydrolysis by eliminating other compounds that may interfere with pectin recovery, resulting in an increased yield and quality. Moreover, the recovered solid (~160 g) from pectin extraction was further hydrolyzed with sulfuric acid. It is worth noting that this solid consisted of ~70 g of the residual biomass and ~89 g of citric acid that remained in this fraction after the pectin production process. A yield of extraction of 63.46 ± 0.95 g/100 g DM was obtained from sulfuric acid hydrolysis, resulting in ~45 g of extract (i.e. the hydrolysate containing sugars, organic acids, and furans) and ~26 g of remaining biomass after the process. The yield of extraction from this process was also higher than that reported by the authors previously [37], which might have been caused by the accumulation of residual sugars from previous steps and changes in the composition of the solid matrix. Nonetheless, these results demonstrated that only around 10 % of the initial sample is left behind after being fractionated sequentially. Moreover, it must be noted that the presence of citric acid and other substances in liquid and solid fractions of the biorefinery could result in undesired effects on further processing of these streams. Pictures of OR and its visual change after each processing step are shown in Fig. S2.

3.2. Characterization of extracts and hydrolysates from orange residues before (raw) and after treatments

The composition of the liquid fractions recovered from the biorefinery is listed in Table 2. The first one is the extract obtained from SLE (Stream 1), rich in glucose and fructose. This extract contains flavanones such as naringin and hesperidin. The extraction was performed using an

Table 3
Compositional analysis of the residual solids obtained after each processing step.

Proximate analysis					
Parameter % (d. w.)	Initial sample	After SD	After SLE	After CA hydrolysis	After SA hydrolysis
Moisture	11.55 ± 0.14	10.67 ± 0.15	10.63 ± 0.09	10.34 ± 0.27	12.81 ± 0.58
Ash (950 °C)	3.33 ± 0.04	3.64 ± 0.01	4.15 ± 0.01	1.13 ± 0.001	0.66 ± 0.03
Volatile Matter	78.61 ± 0.06	77.00 ± 0.05	72.41 ± 0.05	75.63 ± 0.06	73.62 ± 0.06
Fixed Carbon	18.06 ± 0.15	19.36 ± 0.10	23.44 ± 0.23	23.24 ± 0.21	25.72 ± 0.13
Calorific value (kJ/kg)	16675	16419	16242	14149	17710
Ultimate analysis[a]					
Parameter % (d. w.)	Initial sample	After SD	After SLE	After CA hydrolysis	After SA hydrolysis
Carbon	40.62 ± 0.05	41.03 ± 0.05	39.90 ± 0.05	40.21 ± 0.05	42.37 ± 0.05
Hydrogen	5.10 ± 0.05	4.95 ± 0.05	4.64 ± 0.05	4.12 ± 0.05	5.15 ± 0.05
Nitrogen	0.86 ± 0.05	0.82 ± 0.05	0.82 ± 0.05	0.52 ± 0.05	0.62 ± 0.05
Sulfur	0.14 ± 0.05	0.21 ± 0.05	0.10 ± 0.05	0.10 ± 0.05	1.13 ± 0.05
Oxygen	53.28 ± 0.05	51.65 ± 0.05	47.11 ± 0.05	51.86 ± 0.05	44.85 ± 0.05
Compositional analysis					
Parameter % (d. w.)	Initial sample	After SD	After SLE	After CA hydrolysis	After SA hydrolysis
Ash	3.33 ± 0.04	3.64 ± 0.01	4.15 ± 0.01	1.13 ± 0.001	0.66 ± 0.03
Protein	4.31 ± 0.05	5.25 ± 0.05	5.06 ± 0.05	3.38 ± 0.05	4.31 ± 0.05
Extractives	55.78 ± 0.71	54.51 ± 0.39	18.90 ± 0.49	57.11 ± 0.5[b]	23.39 ± 0.05
Lignin	3.94 ± 0.22	4.05 ± 0.19	5.41 ± 0.01	3.70 ± 0.04	6.79 ± 0.01
Hemicellulose	5.73 ± 0.53	5.60 ± 0.55	11.38 ± 1.45	14.73 ± 0.16	19.44 ± 1.72
Cellulose (Glucan)	7.18 ± 0.52	5.59 ± 0.54	12.38 ± 0.27	17.55 ± 0.76	43.21 ± 1.89
Starch (Non-cellulosic Glucan)	1.48 ± 0.01	1.44 ± 0.02	2.71 ± 0.02	N. D.	N. D.
Pectin	22.36 ± 0.84	21.91 ± 0.75	39.6 ± 1.10	3.21 ± 0.16	0.97 ± 0.03
Total	102.63 ± 2.91	102.00 ± 2.45	99.59 ± 3.40	100.82 ± 1.67	98.78 ± 4.24

[a] Dry-ash free.
[b] Including citric acid.

ethanolic solution, which was partially evaporated (~80 %) during sugar concentration. A complete removal of ethanol was not possible since the sugar-rich mixture increased its viscosity upon further concentration, making its manipulation problematic. The citric acid present in the extract (that comes naturally in OR) was partially removed (~75 %) upon treatment as calcium citrate. However, some citric acid still remained in the mixture. Future work could explore further purification of phenolic compounds from Stream 1 using techniques such as supercritical fluid extraction [61–63], enzyme-assisted supercritical fluid extraction [64], or supercritical fluid chromatography [65], enabling selective recovery and higher purity of individual phenolics.

The citric acid hydrolysate used for pectin production caused the release of glucose, xylose, and arabinose. However, as observed in previous work the hydrolysis using citric acid is more selective for the recovery of pectin (as galacturonic acid) and sugars (from structural carbohydrates) are recovered to a less extent. The presence of HMF and acetic acid indicates that part of the released sugars was degraded during the process.

The residual hydrolysate obtained after pectin recovery (Stream 2) was diluted with ethanol during pectin precipitation and then further washed with water. After concentration and treatment of the mixture for citric acid and acetic acid removal, the hydrolysate was rich in xylose, glucose and contained a small amount of arabinose. It was possible to remove ~95 % of acetic acid and ~80 % of citric acid from the stream after their precipitation as calcium citrate and calcium acetate (Fig. S2). However, the evaporation of water also caused that the remaining citric acid in the solution was also concentrated together with sugars. Evaporation appears also to have removed part of the acetic acid and HMF present in the stream.

The sulfuric acid hydrolysate (Stream 3) was also concentrated and treated for the removal of citric acid and acetic acid. In this case, some ethanol that came with solids after pectin recovery (used for washing) remained and ended up in the hydrolysate. As happened with Stream 1, it was not possible to remove entirely ethanol due to the viscosity of the mixture after evaporation. The treatment removed ~80 % of citric acid, ~90 % of acetic acid, and 50 % HMF that were initially present. After treatment, the stream had a high concentration of xylose and arabinose and a small amount of glucose.

3.3. *Characterization of the residual solids obtained after each processing step*

The compositional analysis of OR and the solid fractions recovered after each processing step of the biorefinery is presented in Table 3. In the first process, the overall composition of the biomass changed slightly from the initial sample since SD removed only a small proportion of the total extractives. As a consequence, the values for the proximate analysis, ultimate analysis, and chemical composition were slightly similar.

Regarding the biomass after SLE, it is clear that the process was able to remove around 65 % of the remaining extractives which, as seen in section 3.2., consisted mainly of free sugars (glucose and fructose). As a result, the volatile matter content of the biomass was reduced, together with its calorific value, and the oxygen content. The removal of extractives caused a redistribution of the chemical composition in the solid fraction where pectin came to be the most abundant fraction, and the proportion of cellulose, hemicellulose, and lignin increased. During extraction some soluble proteins and minerals could have been removed.

The composition of the solid obtained after pectin extraction shows how this substance was removed from the biomass. Nonetheless, it must be mentioned that not all the hydrolyzed pectin can be recovered later during precipitation and will remain in the liquid fraction as galacturonic acid. The hydrolysis caused slight release of glucose, xylose, and arabinose but the proportion of structural carbohydrates present in the solid still increased due to pectin removal. It is worth noting that the extractive content increased, which was caused by some part of the citric acid used for hydrolysis that remained in the solid matrix. The presence of citric acid also altered the proximate and ultimate compositions by reducing the calorific value of the biomass and increasing volatile matter and oxygen content. Clearly, the presence of residual citric acid in both liquid and solid fractions is problematic and could alter further valorization of the biomass. More studies are needed to evaluate feasible alternatives for the recovery of citric acid in a biorefinery from citrus residues and how these could be recycled or used in other products.

At last, the solid residue left after sulfuric acid hydrolysis increased in its fixed carbon content, calorific value, and ratio between Carbon and Oxygen. Table 3 shows that the hydrolysis mostly caused the breakdown of hemicellulose, which is in line with the composition of the liquid hydrolysate (Stream 3), rich in xylose and arabinose, as discussed in section 3.2. Besides that, the citric acid that was in the solid from the previous step appeared to have been mostly recovered in the hydrolysate (Table 2) which could result from citric acid decomposition due to the

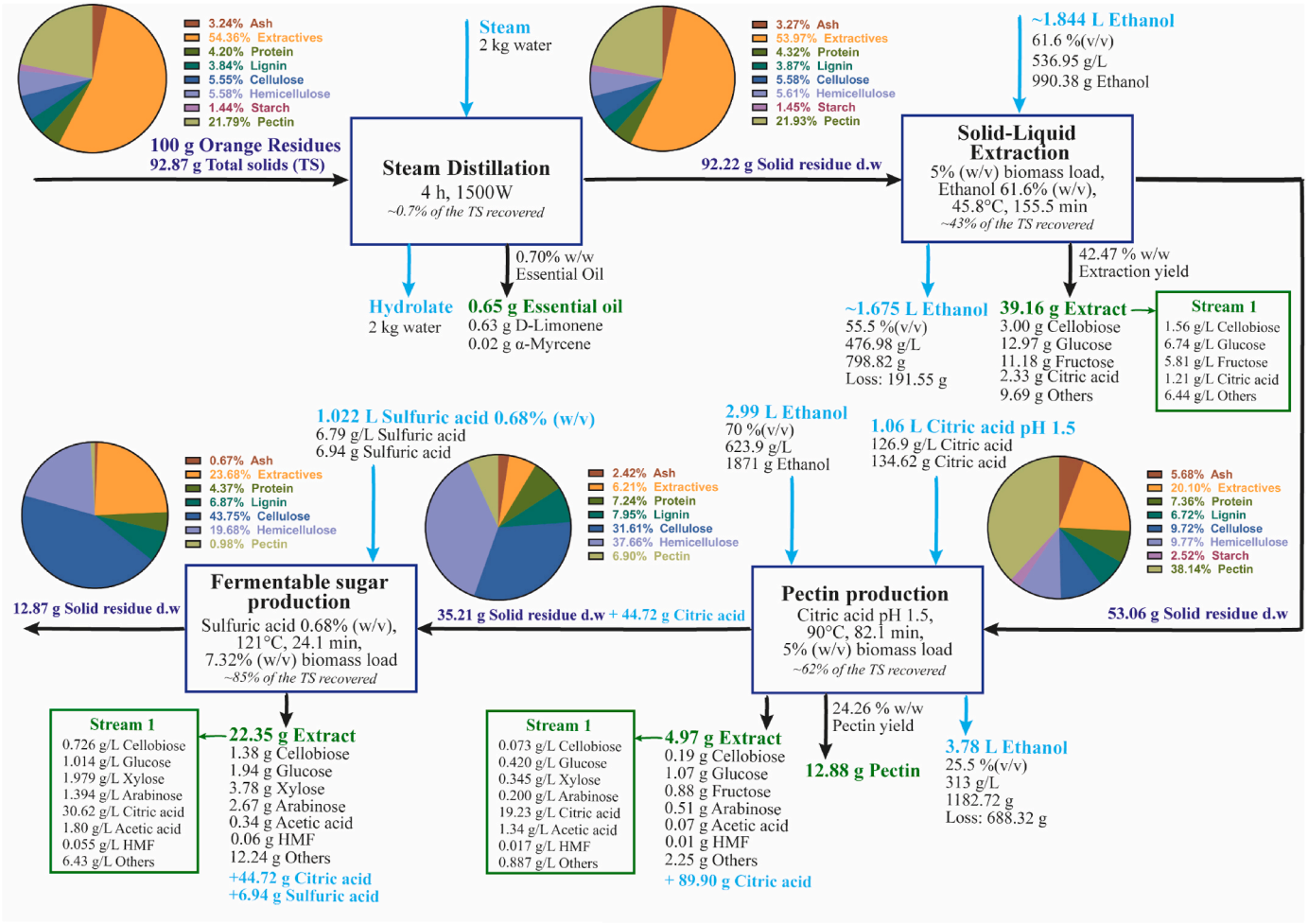

Fig. 2. Mass balance of the cascade biorefinery.

presence of sulfuric acid, releasing carbon monoxide, water and β-ketoglutaric acid [66]. The removal of a high fraction of hemicellulose during sulfuric acid hydrolysis resulted in a higher proportion of cellulose and lignin in the solid residue, which relates to the changes in proximate and ultimate compositions of the solid. Due to its composition, it is possible that this last solid fraction could be further valorized for energy production through thermochemical processes or anaerobic digestion, which is in concordance with the concept of biomass cascading where energy production is always in the last stage of the biorefining sequence.

3.4. Mass balance of the biorefinery cascade

The mass balance of the biorefinery using a calculation basis of 100g of OR is shown in Fig. 2. It is possible to see the amounts recovered of each substance and how the initial biomass is fractionated sequentially. The normalized composition of the residual solids makes evident the changes that occurred to the biomass after each processing step. From the initial total solids, ~0.7 % were recovered in SD, ~43 % after SLE, ~62 % after pectin extraction, and 85 % after sulfuric acid hydrolysis. The reduction on solids along the process demonstrates how biomass cascading maximizes the recovery of substances in the biorefinery, and that the residual solids at the end could still be used. As mentioned in section 2.3, the removal of extractives (during SD and SLE) and pectin (during citric acid hydrolysis) results in an increase on the proportion of cellulose, hemicellulose, and lignin, which leaves the residual solids with a composition more similar to that found in conventional lignocellulosic residues, like sugarcane bagasse.

The diagram also shows the requirements for steam, liquid water, ethanol, citric acid, and sulfuric acid in the biorefinery which could be a valuable input for more detailed design and technoeconomic evaluation. The results obtained in this work show that the removal of citric acid from both liquid and solid streams is critical in a biorefinery from OR and should be studied in more detail in the future to avoid its interference and allow its reuse. Moreover, the ethanol used for solid-liquid extraction and pectin precipitation could be partially recycled again into the process which could reduce costs, which it is important to consider given the amount of ethanol that could be left behind in solid fraction or lost due to evaporation. Furthermore, the water used for extractions can also be recovered during concentration of the sugar-rich streams and recycled into the process. Moreover, since all operations require energy to achieve the conditions required either for producing steam, which could be expensive, or reaching the necessary temperature for solid-liquid extraction or hydrolysis, the integration of residual hot streams could be implemented to enhance energy efficiency. Further evaluation of the integral sustainability of available extraction techniques and biorefinery schemes in orange biomass valorization should be explored in future works.

It is evident that the evaluated biorefinery could be feasible at a technical level and would allow further improvements, like the use of side-streams and the production of energy from the solid residue of sulfuric acid hydrolysis, that could make the evaluated sequence also feasible at an economic and environmental level. What is more, the process is not targeted to one product and since the composition of biomass is considered in each step it achieves the recovery of several value-added substances by minimizing the residual biomass left behind

Table 4
Chemical composition of side streams used for fermentation.

Substance (g/L)	Stream 1	Stream 2	Stream 3
Cellobiose	0.54 ± 0.14	0.79 ± 0.01	5.19 ± 0.40
Glucose	19.99 ± 0.96	14.12 ± 0.31	4.55 ± 0.55
Fructose	20.01 ± 0.60	N. D.	N. D.
Xylose	N. D.	23.06 ± 0.58	17.51 ± 1.38
Arabinose	N. D.	2.82 ± 0.1	17.95 ± 1.39
Total Sugars (Sum)	40.00	40.00	40.00
Citric acid	1.48 ± 0.11	163.11 ± 3.54	52.49 ± 3.54
Acetic acid	0.74 ± 0.01	3.61 ± 0.10	7.06 ± 0.58
HMF	N. D.	0.02 ± 0.01	0.18 ± 0.02
Furfural	N. D.	N. D.	N. D.
Ethanol	66.44 ± 0.52	N. D.	55.15 ± 1.37
TPC	1.07 ± 0.04	0.70 ± 0.01	1.77 ± 0.14
Protein	1.88 ± 0.11	2.02 ± 0.22	3.80 ± 0.14
pH	6.05	7.77	6.13

Stream 1: Solid-liquid extract, Stream 2: Citric acid hydrolysate, Stream 3: Sulfuric acid hydrolysate, N. D.: not detected.

at the end of the process.

3.5. Fermentation of side-streams from the biorefinery

To extract further value from the biorefinery the production of value-added substances through the fermentation of the sugar-rich liquid side-streams that were obtained was performed. The recovered streams after treatments were adjusted to contain 40 g/L of sugars (Sum of C5 and C6 sugars), as shown in Table 4. The composition of the fermentation media shows that substances that could inhibit yeast growth like citric acid, acetic acid, ethanol, HMF, and phenolic compounds are still present. However, it is unknown how much these substances could affect each yeast during fermentation. The partial removal of citric acid and acetic acid helped to adjust the pH of the streams, which was finally adjusted to 5.8 for fermentation in all cases. Moreover, the protein content measured in the streams that is caused by the release of soluble proteins and amino acids could serve as a nitrogen source for the yeasts. It is important to mention that the fermentation experiments using synthetic media contained the same sugar composition for each stream, as shown in Table 4, but without the inhibitors.

3.5.1. Effect of fermentation media and nutrients in the production of value-added substances

The use of the treated streams as fermentation media resulted in growth inhibition in all cases for *K. marxianus* NRRL Y-6373 and *H. polymorpha* CBS 4732. It is possible that the presence of ethanol in Streams 1 and 3, and citric acid in Streams 2 and 3 could have interfered with yeast growth. Moreover, *S. cerevisiae* Ethanol Red was only able to grow in Stream 1 and was inhibited in Streams 2 and 3 possibly due to the high concentration of citric acid in those media.

In Fig. 3 it is possible to see that *S. cerevisiae* Ethanol Red grew in a

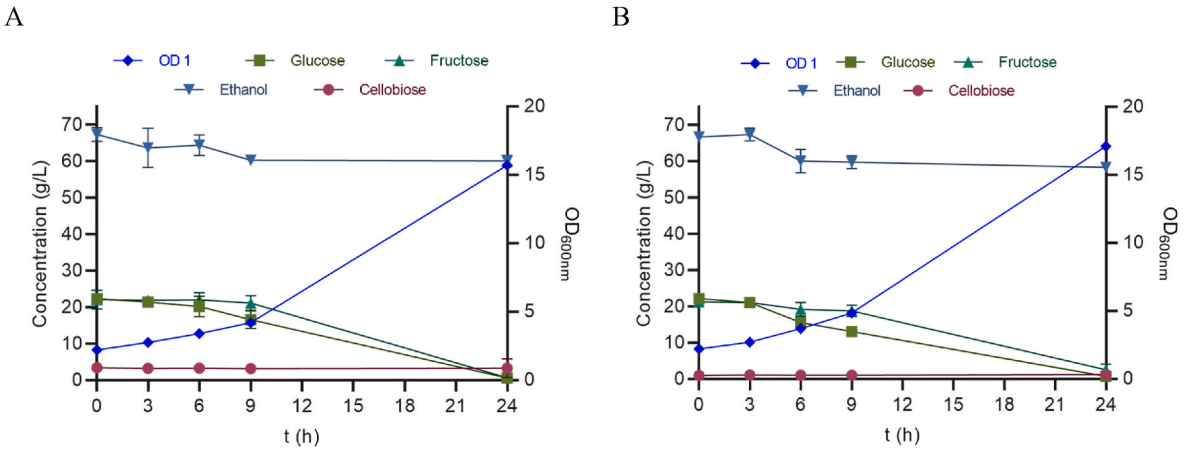

Fig. 3. Growth and substrate consumption of *S. cerevisiae* Ethanol Red in Stream 1 (SLE extract) with (A) and without nutrients (B). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

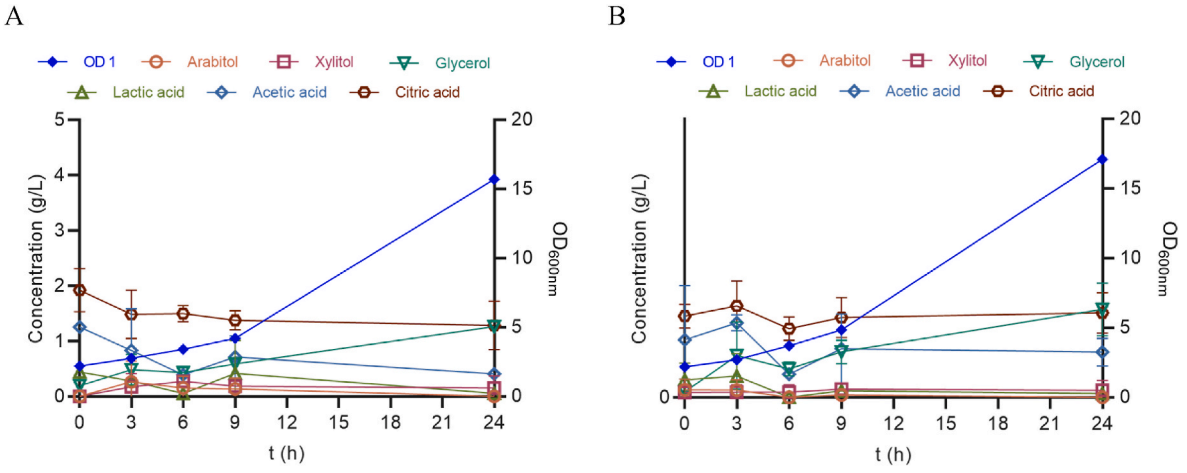

Fig. 4. Growth and metabolite production of *S. cerevisiae* Ethanol Red in Stream 1 (SLE extract) with (A) and without nutrients (B). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

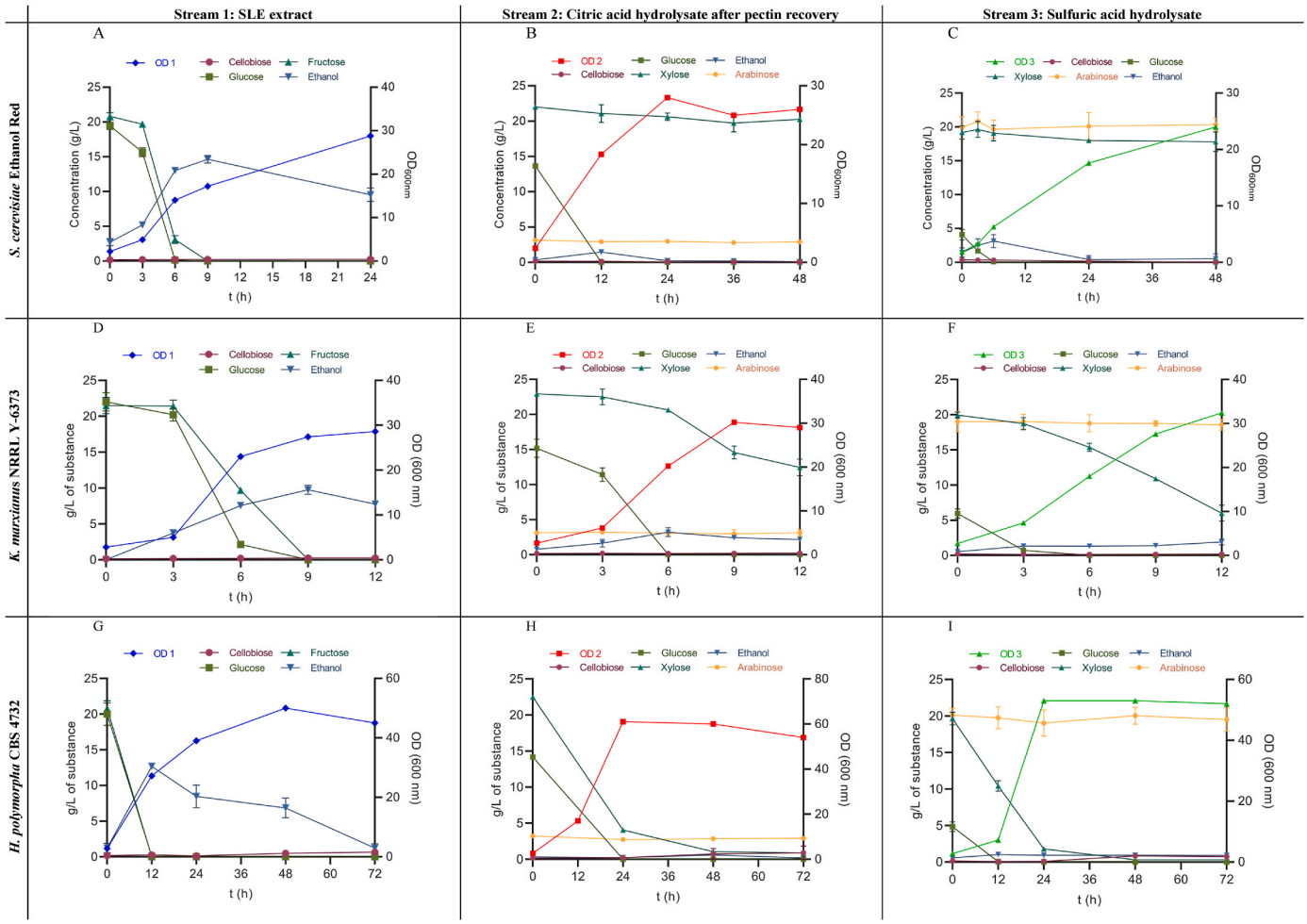

Fig. 5. Yeast growth, sugar consumption, and ethanol production in synthetic media without inhibitors.

similar way in Stream 1 with (Fig. 3A) and without nutrients (Fig. 3B), reaching an OD_{600} value ~16. This behavior could indicate that the use of nutrients in the media had not significative effect. Besides that, it appears that the yeast used not only fructose and glucose as carbon sources (completely consumed after 24 h) but also consumed ethanol, reducing its concentration from ~66 g/L down to ~60 g/L. *S. cerevisiae* Ethanol Red has been known to have high ethanol tolerance [67], which is in line with the in the growth pattern and the ethanol consumption observed in Fig. 3. During the process *S. cerevisiae* slightly consumed some citric acid (from ~1.5 g/L to 1.3 g/L) and acetic acid (from ~0.75 g/L to ~0.45 g/L) present in the media and released ~1.2 g/L of glycerol (Fig. 4). The presence of ethanol and citric acid had an evident negative effect in yeast growth and interfered with the fermentation process of all yeasts. For the valorization of the sugars present in the side-streams it is necessary to remove both inhibitors to an extent to which they do not interfere with fermentation.

The results for the growth in synthetic media (Figs. 5 and 6) allowed to see how yeasts would behave in ideal conditions where all inhibitors are removed. It is worth remembering that Stream 1 contained ~20 g/L of glucose and fructose, Stream 2 had ~23 g/L of xylose, ~14 g/L of glucose, and ~2.8 g/L of arabinose, and Stream 3 had ~17.5 g/L of xylose and arabinose, and ~4.5 g/L of glucose.

In the case of *S. cerevisiae* Ethanol Red, glucose, and fructose consumption during fermentation (Fig. 5A) was used to produce biomass (OD_{600nm} of 29, at 24h) and ethanol. At 9h, a maximum ethanol concentration of 13.04 ± 0.4 g/L (0.326 g/g sugars) was obtained together with 1.24 ± 0.06 g/L of glycerol and less than 0.5 g/L of acetic acid (Fig. 6A). After that time, the yeast started to use ethanol as carbon source which allowed its growth to continue. In contrast, the higher xylose content compared to glucose in Streams 2 and 3 (Fig. 5B and C) resulted in low ethanol productions (at 9h) with 1.24 ± 0.06 g/L and 3.14 ± 0.06 g/L, respectively. After glucose was entirely consumed (after 12 h for Stream 2, and 6h for Stream 3), it is apparent that the yeast started to consume xylose to produce yeast biomass with OD_{600nm} values ~25. There is no indication from the results that arabinose was consumed by *S. cerevisiae* Ethanol Red.

For *K. marxianus* NRRL Y-6373, the use of Stream 1 (Figs. 5D and 6D) resulted in 9.78 ± 0.63 g/L of ethanol at 9h, 0.22 ± 0.02 g/L of xylitol and 0.74 ± 0.02 g/L of glycerol at 6h, and less of 0.5 g/L of acetic acid. The yield of ethanol in this case was 0.245 g/g sugars. These substances' production coincides with the complete consumption of glucose after 6h and fructose after 9h. After 12h, an OD_{600nm} of 28.6 was reached and the ethanol concentration started to decrease. In streams 2 and 3 *K. marxianus* (Fig. 5E and F, and 6E-F) produced more xylitol with 2.26 ± 0.06 g/L (0.098 g/g xylose) and 1.59 ± 0.06 g/L, respectively (0.090 g/g xylose). In contrast to Stream 1, only 3.22 ± 0.62 g/L of ethanol (0.228 g/g glucose) were obtained for Stream 2 at 6h, with 0.41 ± 0.11 g/L of glycerol and 0.98 ± 0.11 g/L of acetic acid. Stream 3 had the lowest ethanol concentration with just 1.90 ± 0.40 g/L (0.418 g/g glucose) and 0.29 ± 0.02 g/L of glycerol. *K. marxianus* NRRL Y-6373 has been reported to produce simultaneously ethanol and xylitol [46,49]. However, under the evaluated conditions Stream 1 only favored ethanol production, which can be related to the absence of xylose in this media. Since Streams 2 and 3 produced some xylitol, it is possible that longer fermentation times could result in higher xylitol yields mainly because xylose was still being consumed at the end of the fermentation.

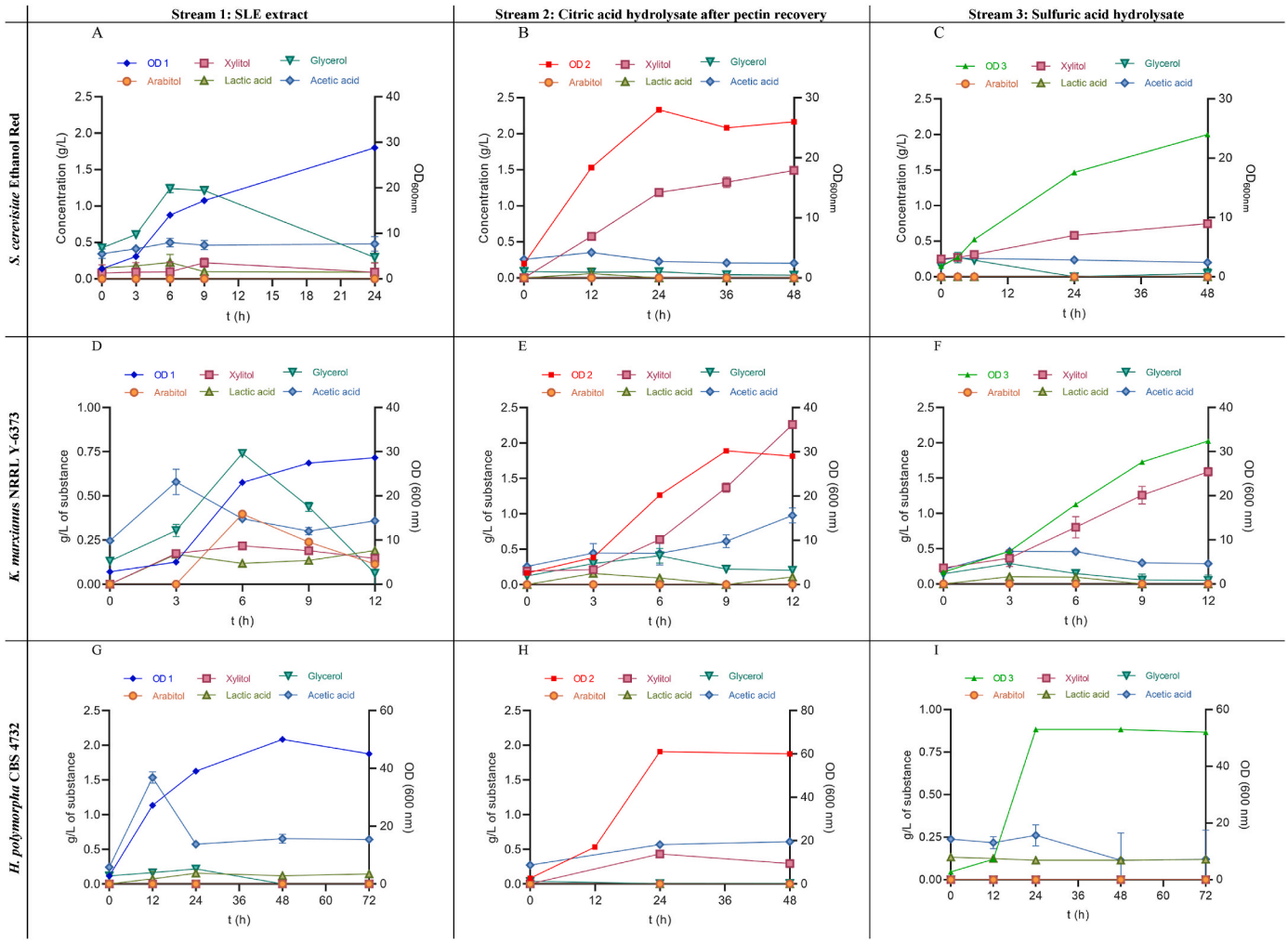

Fig. 6. Yeast growth and metabolite production in synthetic media without inhibitors.

K. marxianus consumed ~25 % of the initial xylose in Stream 2 and 75 % in Stream 3. From these results, it is evident that *K. marxianus* NRRL Y-6373 can use glucose, fructose, and xylose not only to produce ethanol and xylitol, but also to increase yeast biomass achieving similar OD_{600nm} (Between 29 and 33) to those observed in Stream 1. There is no evidence that arabinose was consumed by *K. marxianus* NRRL Y-6373.

Regarding *H. polymorpha* CBS 4732, all glucose and fructose were consumed from Stream 1 (Fig. 5G) in 12 h, resulting in 12.68 ± 0.12 g/L of ethanol (0.317 g/g sugars). After 12h, the yeast started to use ethanol as a carbon source, which resulted in an increase of biomass up to an OD_{600nm} of 50 at 48 h of fermentation. At 72 most of the ethanol was consumed by the yeast and the OD_{600nm} started to decrease indicating the start of the death phase. For Streams 2 and 3 (Fig. 5H and I), only 0.60 ± 0.06 g/L and 1.01 ± 0.11 g/L of ethanol were produced. The production of other substances like glycerol and acetic acid was in the same order of magnitude (Fig. 6G–I). However, the OD_{600nm} of the samples increased up to 61 in Stream 2, and 53 in Stream 3, which shows that *H. polymorpha* CBS 4732 used glucose and xylose to produce biomass, which could be the reason why this this yeast has been reported as promising candidate to produce single-cell protein [48,49]. The yeast consumed all xylose from Streams 2 and 3 after 48h, but it did not consume any arabinose.

The results obtained for the fermentation of the side-streams using conventional and non-conventional yeasts indicate that the production of ethanol could be an interesting option either with *S. cerevisiae* Ethanol Red or *H. polymorpha* CBS 4732, which achieved similar ethanol concentrations and yields from Stream 1 due to the abundance of glucose and fructose. However, *S. cerevisiae* Ethanol Red was slightly more efficient and required 3h less to achieve the same amount of ethanol. Regarding Streams 2 and 3, the most promising alternative is to use it for the production of single-cell protein with *H. polymorpha* CBS 4732 since it would allow to produce value from both C5 and C6 sugars present in those side-streams. The use of *K. marxianus* NRRL Y-6373 could be not ideal since the amount of ethanol and xylitol obtained in any of the streams is low compared to the other yeasts evaluated in our work and the reported data from other xylitol-producing yeasts [48,68]. Moreover, since any of yeasts were able to consume arabinose, it would be necessary to study in future works how this residual fraction could be valorized.

3.5.2. *Effect of inhibitors in fermentation*

The potential integration of the fermentation processes with yeasts into the biorefinery from OR depends highly on the removal of inhibitors to achieve maximum yields. For that reason, it was explored if the presence of ethanol and citric acid could have caused the inhibition seen in the yeasts. Fig. 7 shows the results of growth for each yeast in simulated synthetic media with inhibitors.

S. cerevisiae Ethanol Red (Fig. 7A and B) was able to grow in synthetic Stream 1 with inhibitors, with a shorter lag phase compared to the natural media with and without nutrients. Nonetheless, after 24h a similar OD_{600nm} was reached by both synthetic and natural media from Stream 1, and it is apparent that the yeast could continue to grow. As

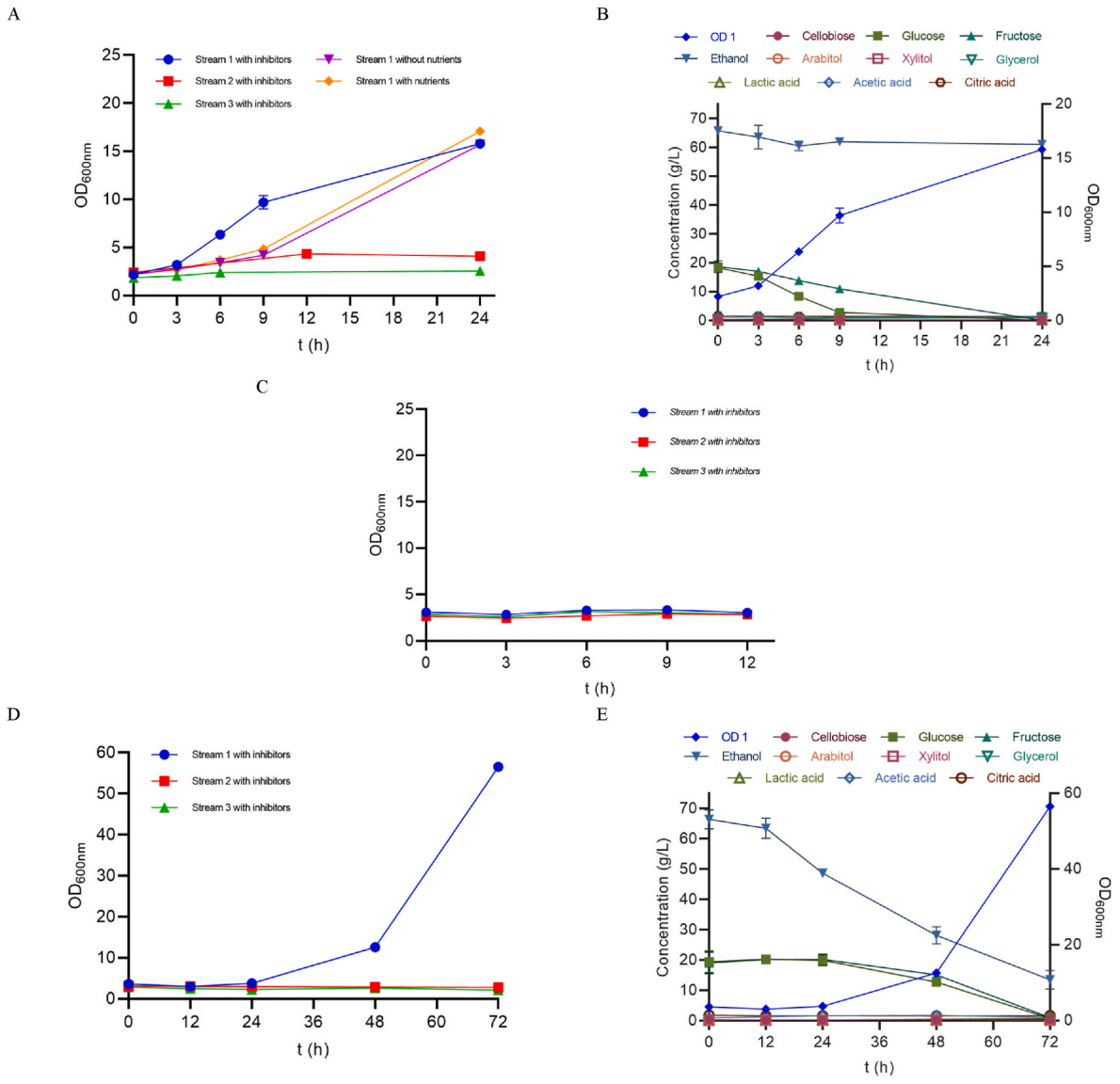

Fig. 7. (A, B) Growth and metabolite production of *S. cerevisiae* Ethanol Red in synthetic media with inhibitors and in the raw SLE extract, (C) Growth of *K. marxianus* NRRL Y-6373 in synthetic media with inhibitors, and (D,E) Growth and metabolite production of *H. polymorpha* CBS 4732 in synthetic media with inhibitors. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

seen earlier, *S. cerevisiae* Ethanol Red can tolerate a high ethanol concentration of ~66 g/L. Fig. 7B shows that *S. cerevisiae* Ethanol Red consumed around 10 % of the initial ethanol present in synthetic Stream 1, showing similar results to those observed in natural media (Fig. 3). On the contrary, the yeast was not able to grow in synthetic Streams 2 and 3, indicating that the citric acid concentration in those media (>50 g/L) could be affecting the fermentations.

Moreover, the use of synthetic media with inhibitors for *K. marxianus* NRRL Y-6373 (Fig. 7C) resulted in complete inhibition in all cases, as seen in section 3.4.1. It is possible that both citric acid and ethanol, with concentrations higher than 50 g/L in both cases, could have been the cause of the inhibitory effect.

In the case of *H. polymorpha* CBS 4732 (Fig. 7D), the yeast grew in synthetic media with inhibitors. It had a long lag phase of ~24 h, which could explain why no growth was seen in the initial experiments with natural media of Stream 1 (Section 3.4.1). It is apparent that *H. polymorpha* CBS 4732 was able to resist ethanol at concentrations ~66 g/L, reaching an OD_{600nm} value of 56.5 after 72h and consuming

Table 5
Qualitative results of growth for the yeasts in different culture media.

	Stream 1				Stream 2				Stream 3			
Yeast	WN	WoN	WI	WoI	WN	WoN	WI	WoI	WN	WoN	WI	WoI
S. cerevisiae Ethanol Red												
K. marxianus NRRL Y-6373												
H. polymorpha CBS 4732												

WN: With nutrients, WoN: Without nutrients, WI: Synthetic media with inhibitors, WoI: Synthetic media without inhibitors.

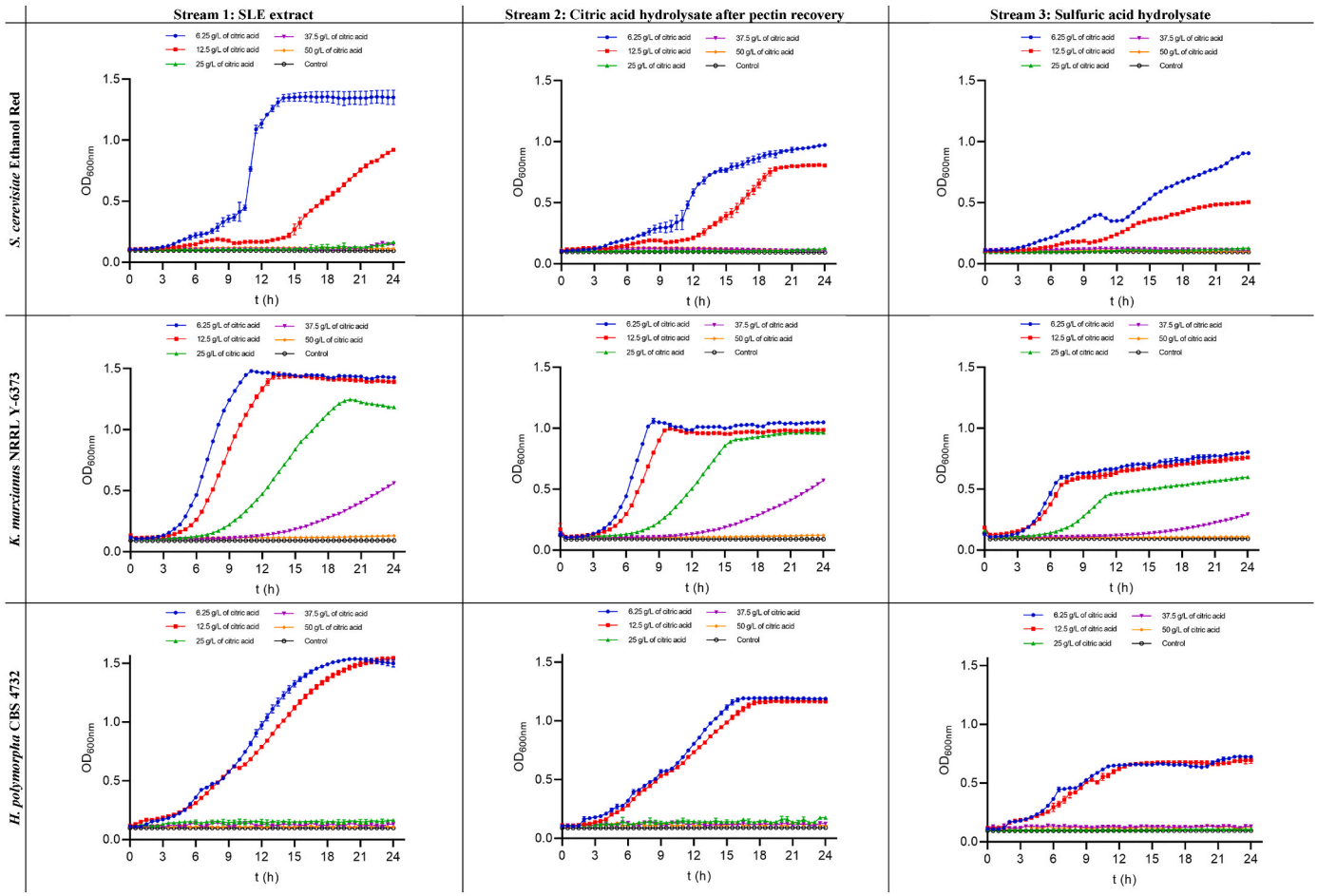

Fig. 8. Minimum inhibitory concentration tests for citric acid using simulated media.

~80 % of the initial ethanol present in the media (Fig. 7E). However, *H. polymorpha* was also not able to grow in Streams 2 and 3, which had citric acid concentrations higher than 50 g/L.

The qualitative results of growth from the fermentation experiments are summarized in Table 5. It is possible to see that the best candidate for ethanol production using Stream 1 could be *S. cerevisiae* Ethanol Red since it is able to tolerate the presence of ethanol, consumes all the sugars present in the stream and under conditions where the inhibitors are not present it is able to produce ethanol at the highest concentration between the all the evaluated yeasts. *K. marxianus* NRRL Y-6373 would not be an ideal candidate since it was inhibited when grown in the treated streams (with and without nutrients) and in synthetic media with inhibitors. Also, when grown in synthetic media without inhibitors, it could consume only a part of the sugars present in the streams and achieved low ethanol and xylitol concentrations. In the case of *H. polymorpha* CBS 4732, ethanol was produced from Stream 1 using synthetic media with and without inhibitors and was able to consume all the sugars present in those media. For Streams 2 y 3, *H. polymorpha* CBS 4732 grew in media without inhibitors, showing that in conditions where citric acid is removed from the hydrolysates, this yeast can consume all glucose and xylose to produce single-cell protein.

Apart from the inhibitory effect of ethanol and citric acid in the yeasts it is worth mentioning that the presence of phenolic compounds in the streams could also interfere with fermentation and its influence should further studied to improve overall yields of desired products. From the sugar-rich streams of the biorefinery, the TPC of Stream 1 (from SLE) appears to be directly related to the polyphenols that are naturally present in OR, like naringin and hesperidin. These flavanones have been reported to competitively inhibit the activity of the hydrolases (α-glucosidase and β-fructosidase) produced by yeasts [69] due to the presence of sugar groups (glycones) attached to those flavonoids. The TPC measured in Stream 2 (residual citric acid hydrolysate) and Stream 3 (sulfuric acid hydrolysate) could be more related to the phenolic acids, phenolic aldehydes, phenolic ketones, and phenolic alcohols that are released as products from acid hydrolysis [70]. It is well known that the type of polyphenols, the functional side groups that contain, and their concentration in the hydrolysates affects how much yeasts can tolerate these substances [71]. It is possible that the presence of flavanones in Stream 1, which only affect hydrolase enzymatic activity, could have not interfered with fermentation since the media consists of already available sugars that were previously obtained from hydrolysis. Also, the TPC of Streams 2 and 3 is low compared with the inhibitory concentration that has been reported for some lignin-derived phenolic compounds that are released from hydrolysis like vanillic acid (3 g/L), syringic acid (3.8 g/L), and p-hydroxybenzoic acid (5 g/L) in fermentations with *S. cerevisiae* Ethanol Red [70]. Future studies should consider the potential interference of reducing sugars, particularly xylose and fructose, in TPC quantification [72,73]. This issue can be addressed by using the acidic Folin reaction, which preserves the stability of phenolic compounds and has shown promise as an effective way to avoid interference in high-sugar matrices [74].

3.5.3. Minimum Inhibitory Concentration (MIC)

The influence of citric acid and ethanol in fermentation was obtained from the results of the Minimum Inhibitory Concentration (MIC) tests. In Fig. 8 it is possible to see how the presence of citric acid had inhibitory effects for all the yeasts. In the case of *S. cerevisiae* Ethanol Red, growth was completely inhibited at 25 g/L of citric acid (Fig. 8). *K. marxianus*

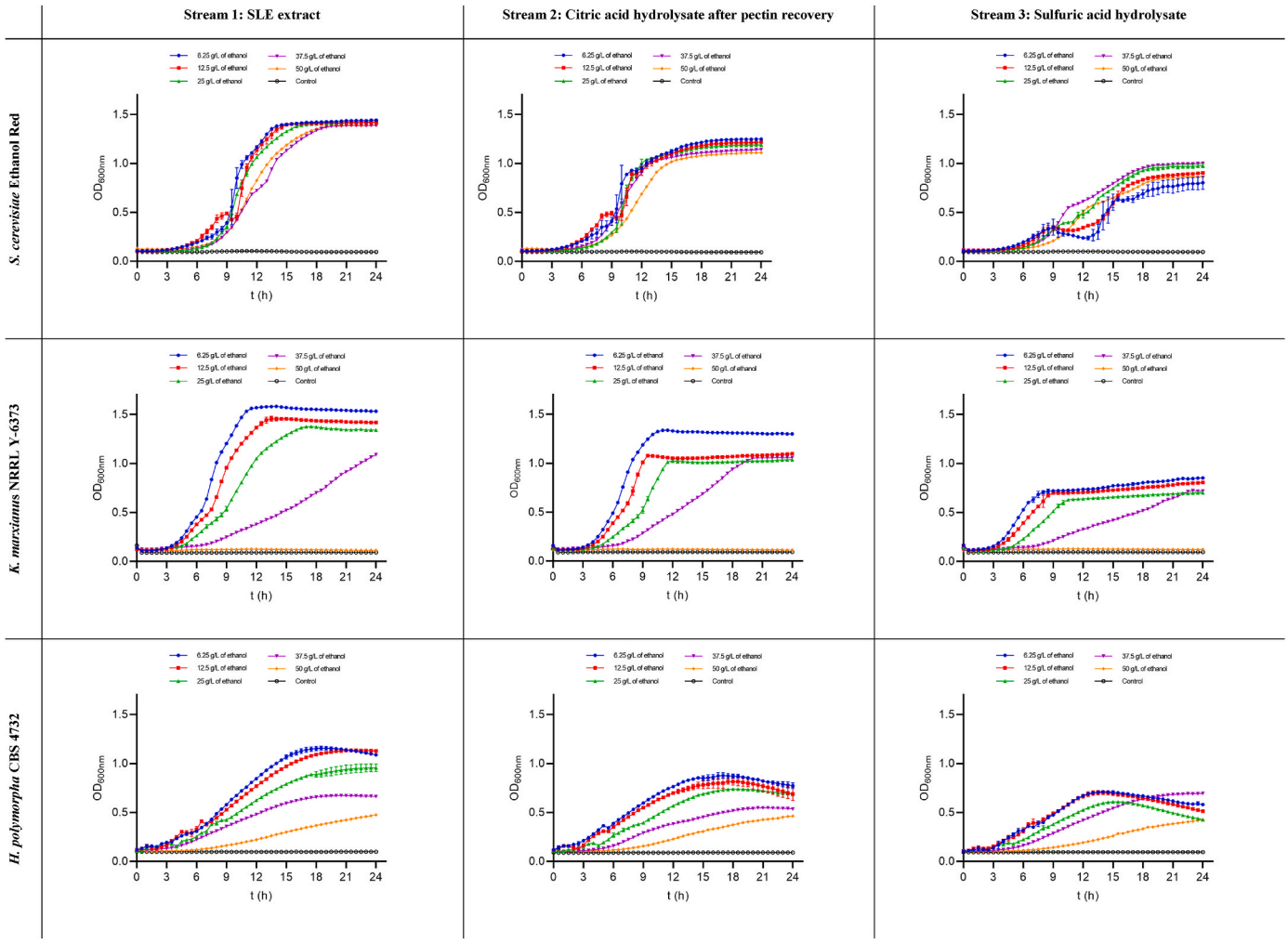

Fig. 9. Minimum inhibitory concentration tests for ethanol using simulated media.

NRRL Y-6373 had more resistance to the presence of citric acid and was inhibited at 37.5 g/L. *H. polymorpha* CBS 4732 was also affected by the presence of citric acid and was inhibited also at 25 g/L. In all cases, the concentration of citric acid was lower than the one measured in Streams 2 and 3, which would explain why the yeasts were not able to grow in those media.

Regarding the MIC of ethanol, this substance did not interfere with the growth of *S. cerevisiae* Ethanol Red (Fig. 9) in any of the concentrations tested, which is line with the ethanol resistance observed in the fermentation experiments and in the literature [67].). *K. marxianus* NRRL Y-6373 resisted up to 37.5 g/L of ethanol and was completely inhibited at 50 g/L, which could also explain why this yeast did not grow in Streams 1 and 3. *H. polymorpha* CBS 4732 was able to grow in all the ethanol concentrations that were tested, which demonstrates the ability of this yeast to tolerate the presence of ethanol up to 50 g/L as happened in the experiments using synthetic Stream 1 with inhibitors.

The results from the MIC tests allowed to identify how much each yeast is affected by the presence of citric acid and ethanol in the media. This data is a valuable input for knowing how much of these substances need to be removed during detoxification treatments. Also, Figs. 8 and 9 show in all cases how the differences in the sugar content of the streams influence yeast growth. All yeast grew better in Stream 1 due to the presence of glucose and xylose, and its growth decreased in Stream 2 which was rich in xylose, and Stream 3 with a high content of xylose and arabinose.

4. Conclusions

The sequential valorization of OR allowed the recovery of valuable substances and reduced the amount of solid biomass left behind from the biorefinery, with approximately 85 % of the initial biomass being extracted/transformed into products. Additionally, the left-over residue could still be used for energy production. In the design of biorefineries from fruit residues, it is important to monitor the compositional changes throughout the process, in both solid and liquid streams, since this allows to obtain a complete mass balance that shows how biomass is affected by the process and how the substances used for extraction interact and affect further processing. It was observed that the use of ethanol and citric acid, which are necessary in the biorefinery from OR, resulted in residual amounts of these substances in both liquid and solid fractions. The complete removal of citric acid and ethanol from the streams is difficult and should be studied in detail in future works since it interferes with other valorization techniques causing undesired effects. This was evidenced when the sugar-rich streams were fermented using *S. cerevisiae* Ethanol Red, *K. marxianus* NRRL Y-6373, and *H. polymorpha* CBS 4732 which were inhibited by either citric acid or ethanol. However, if these inhibitors are sufficiently removed, it is possible that the stream obtained from SLE (rich in glucose and fructose) could be used for ethanol production with *S. cerevisiae* and *H. polymorpha*, and the streams obtained from acid hydrolysis (containing C5 and C6 sugars) could be used to produce single-cell protein with *H. polymorpha*. Nonetheless, in the studied biorefinery a variety of substances were

extracted without focusing on a sole product, which could increase the feasibility of the proposed sequence and opens the possibility to incorporate other valorization techniques.

CRediT authorship contribution statement

D.D. Durán-Aranguren: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **C.K. Yamakawa:** Validation, Methodology, Investigation. **J. Ordeñana:** Methodology, Investigation. **R. Sierra:** Writing – review & editing, Supervision. **J.A. Posada:** Writing – review & editing, Supervision. **S.I. Mussatto:** Writing – review & editing, Supervision, Resources, Project administration, Conceptualization.

Declaration of competing interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biombioe.2024.107514.

Data availability

Data will be made available on request.

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