Phenolic compounds modulation in β-farnesene fed-batch fermentation using sugarcane syrup as feedstock

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Abstract

Sugarcane syrup is the feedstock used in the industrial production of β-farnesene through *Saccharomyces cerevisiae* fermentation. However, it contains phenolic compounds, which may interfere with yeast performance. The aim of this work was to modulate the transfer of phenolic compounds from sugarcane syrup to the broth, throughout 13 days of β-farnesene fed-batch fermentation in 2-L bioreactors. This was accomplished by applying two mathematical calculations: a mass balance and the Weibull kinetic model. The concentrations of most phenolic compounds increased until day 3 of fermentation and then remained constant until the end of the process. However, quantities of hydroxybenzaldehyde, protocatechuic, caffeic, ferulic and *p*-coumaric acids, decreased after day 2. The Weibull model exhibited better fit to the data, with an R^2 of 0.85 or higher. This work provides for the first time a model describing phenolic accumulation in β-farnesene fermentation, which can be a valuable tool to apply in similar processes.

Keywords: sugarcane syrup; phenolic compounds; *Saccharomyces cerevisiae*; β-farnesene; fed-batch fermentation.

1. Introduction

Sugarcane syrup is a product of the sugarcane (*Saccharum officinarum* L.) industry, resulting from the crushing of peeled sugarcane, followed by the evaporation of the juice obtained in the crush (Abdel-Aleem, 2020). Sugarcane is mainly grown in tropical locations, such as India and Brazil, and its processed syrup can be used for human consumption and in the production of value-added molecules through fermentation. Syrup contains highly concentrated sugars (from 33 to 75 %) in the form of sucrose, glucose and fructose, is low cost, and is produced from a renewable source (Abdel-Aleem, 2020). All these characteristics contributed to its application as carbon feedstock in industrial fermentations with engineered yeast *Saccharomyces cerevisiae*, and an example of that is the production of the terpenoids β-farnesene, artemisinin, and patchoulol (Carsanba et al., 2021; Paddon et al., 2013).

In the industrial production of terpenoids, the mode of fermentation employed is fed-batch since it combines the advantages of both batch and continuous fermentation modes (Mesquita et al., 2019; Zabed et al., 2014). It avoids high substrate concentrations, with lower inhibition from the Crabtree effect. Furthermore, this operating mode is superior to the other two as it can present high cell density, extended cell lifespan, maximum cell viability and more control over oxygen saturation. All these factors result in the increase of product formation, and lead to high yield and productivity (Zabed et al., 2014).

However, the fact that sugarcane syrup is not a pure form of substrate implies that other substances, which come from the sugarcane plant, are also supplied to the microorganisms during feeding. Phenolic compounds are an

example of these molecules. In fact, in the broth of fed-batch fermentations, the concentrations of these compounds may be increasingly high because the feedstock is added over time, instead of being supplied in a limited and single quantity in the beginning of the culture, as is done in the batch mode (Qureshi et al., 2008). Phenolic compounds, also known as phenols or polyphenols, are secondary metabolites of plants, involved in their defense against pathogens (Huang et al., 2018). These compounds can influence a fermentation process in a positive way, by controlling the reactive oxygen species (ROS), or in a negative way, by exerting antimicrobial activity (Adeboye et al., 2014; Baptista et al., 2020; Duarte-Almeida et al., 2011).

During aerobic growth of microorganisms, which includes yeast fermentation, there is the formation of free radicals and other ROS, due to the contact between oxygen and organic compounds. ROS, which are oxidant agents, accumulate during the lifetime of a cell, cause oxidative stress and promote cell aging (Sadowska-Bartosz & Bartosz, 2014). Many phenolic compounds show capacity to neutralize ROS, indicating that they have antioxidant capacity (Hur et al., 2014; Murkovic, 2003). However, they have also antimicrobial activity, which is not desired in microbial fermentations because these molecules may inhibit cell growth, reduce sugar consumption and lead to poor productivity (Adeboye et al., 2014). In fact, some molecules inhibit strongly the growth of *S. cerevisiae*, which is the case of *p*-coumaric acid (96 % inhibition at 1200 mg/L), ferulic acid (76 % inhibition at 1000 mg/L), caffeic acid (total inhibition at 3600 mg/L) or *p*-hydroxybenzoic acid (total inhibition at 2700 mg/L) (Gu et al., 2015; Merkl et al., 2010). Many phenolic compounds cause

inhibition through damaging the integrity of the cell membrane, affecting the ability of the membrane i) to function as a selective barrier, by allowing inhibitory molecules to pass through, and ii) to function as an enzyme matrix, by conditioning the support for the anchoring of enzymes (Adeboye et al., 2014; Kim et al., 2013; Zhang et al., 2016).

Since phenolic compounds that are present in sugarcane syrup may impact the industrial fermentation performance, the current work has as specific aims to: i) characterize the phenolic content of sugarcane syrup used in the industrial production of β-farnesene; ii) characterize the evolution of phenolic content in 13 days β-farnesene fed-batch fermentations with engineered *S. cerevisiae*; iii) apply two different mathematical calculations (a theoretical mass balance and the Weibull kinetic model) to describe the transfer of phenolic compounds from this feedstock to the culture broth, throughout the fermentation time.

2. Materials and Methods

2.1 Sugarcane syrup characterization

The sugarcane syrups studied in this work were two batches obtained from Amyris Inc. (a Biotechnology Company with presence in Brazil), with both batches being produced from sugarcane harvested in the beginning of spring in Brazil (September). These syrups were characterized regarding total reducing sugars (TRS) content, density, pH, individual phenolic compounds and total phenolic content (TPC). Triplicate of analysis was used. TRS content was

determined using Ion Chromatography High-Performance Anion-Exchange Chromatography coupled with Pulse Electrochemical Detection (IC HPAE-PAD).

The analysis of all phenolic compounds was performed by Liquid Chromatography – Electrospray Ionization – Ultrahigh-Resolution - Quadrupole Time of Flight – Mass Spectrometry (LC-ESI-UHR-QqTOF-MS) (Oliveira et al., 2015). The separation was performed in a Bruker Elute series equipped with a UHR-QqTOF mass spectrometer (Impact II, Bruker Daltonics, Bremen, Germany) and a BRHSC18022100 intensity Solo 2 C18 column (100 × 2.1 mm, 2.2 µm, Bruker). Separation was carried out at a flow rate of 0.25 mL/min with the following elution gradient: 0 min, 0 % B; 10 min, 21.0 % B; 14 min, 27 % B; 18.30 min, 58 %; 20.0 min, 100 %; 24.0 min, 100 %; 24.10 min, 0 %; 26.0 min, 0 % and mobile phase A (0.1 % aqueous formic acid) and B (acetonitrile with 0.1 % formic acid). The MS acquisition was set using negative ionization mode with the selected parameters: end plate off set voltage, 500 V; capillary voltage, 3.0 kV; drying gas temperature, 200 °C; drying gas flow, 8.0 L/min; nebulizing gas pressure, 2 bar; collision radio frequency (RF), from 250 to 1000 Vpp; transfer time, from 25 to 70 µs; collision cell energy, 5 eV. The internal mass calibration used sodium formate clusters.

The elemental composition was confirmed according to accurate mass and isotope rate calculations designated mSigma (Bruker Daltonics) and phenolic compounds were identified based on its accurate mass $[M-H]^-$. Vitexin, diosmetin, naringenin, isoschaftoside, orientin, viexin-2-O-rhamnoside (Extrasynthése, France), tricin, naringenin-7-O-glucoside, luteolin,

protocatechuic acid, vanillic acid, *p*-coumaric acid, caffeic acid, ferulic acid, chlorogenic acid, gentisic acid, 4-hydroxybenzaldehyde, 4-hydroxybenzoic acid, 3,4-dihydroxybenzalhedyde, syringic acid (Sigma-Aldrich) were used as external standards and the results were expressed in mg/L.

2.2 Microorganisms and inoculum preparation

S. cerevisiae used in this study was a recombinant strain that is a precursor of the yeast strains that have been used for farnesene manufacturing in recent years by Amyris, Inc. This strain presents constitutive and stable expression of the β-farnesene production pathway. In this study, the Amyris industrial fermentation process was simulated, from seed growth to the production fermentation. This process consists of two seed-flask steps, two batch bioreactor steps and main fed-batch bioreactor fermentation.

The yeast was activated from two cryovials (each with 1 mL of glycerol stock culture), in an Erlenmeyer flask with 65 mL of culture medium (inoculum of 3 %) and incubated for approximately 46 h. After that, 150 mL medium of second seed flask was inoculated with 12 mL of the first seed flask culture (inoculum of 8%) and incubated for approximately 23 h. The culture medium of seed flasks contained trace metals, vitamins (according to van Hoek et al. (2000)), ammonium phosphate monobasic (7 g/L), potassium phosphate monobasic (1 g/L), magnesium sulfate heptahydrate (0.5 g/L), yeast extract (5 g/L), succinate buffer at pH 5.0 (6 g/L) and sucrose (70 g/L). After inoculation, the seed flasks were incubated at 30 °C and 200 rpm in an incubator (Eppendorf, New Brunswick™ Innova® 44/44R) with 5 cm of orbital diameter.

After two seed flask steps, 1 L medium of the first step of batch bioreactor was inoculated with 2 mL of the second seed flask culture (inoculum of 0.2%). Then, 65.2 mL inoculum from the first step batch bioreactor was used to inoculate 1.2 L culture of second batch bioreactor (inoculum of 4.2%). The batch bioreactor steps were incubated for approximately 48 hours in a reactor (Eppendorf, reference 76DR03F) with 2.7 L of working volume. Process conditions were set at a temperature of 30 °C, pH of 5.0 (regulated by using a solution of 25 % ammonium hydroxide) and dissolved oxygen (DO) percentage of at least 30 % (regulated by agitation ramp ranging between 300 rpm and 1200 rpm). The airflow was 0.5 L/min in the first and 0.75 L/min in the second seed bioreactor. The first seed step bioreactor medium contained 1 L of culture medium with trace metals, vitamins, ammonium phosphate monobasic (7 g/L), yeast extract (2 g/L), 70 g/L of TRS provided with Brazilian sugarcane syrup and Tergitol L-81 as an anti-foam agent. The second seed bioreactor step consisted of 1.5 L of culture medium containing trace metals, vitamins, 160 g/L of sugarcane syrup TRS and Tergitol L-81.

2.3 Fed-batch fermentation

After the two seed bioreactor passages, the main fermentation process was performed according to Meadows et al. (2016). The bioreactors, containing a final volume of 1.2 L of culture media with 3 g/L of ammonium phosphate monobasic, the same trace metals and vitamins solutions as in previous steps and 10 g/L of sugarcane syrup TRS, were inoculated with 353 mL of inoculum from the second reactor step (inoculum of 29.4 %). Four bioreactor

fermentations were conducted, with two independent runs using syrup batch A and two runs using batch B. The results were presented as the average of 4 independent fermentations. The main fermentation process was performed as a fed-batch fermentation, since sugarcane syrup was fed to the yeast culture throughout the process, according to a feedback-controlled pulse feeding algorithm, in the form of pulses (Meadows et al., 2016). In summary, once all sugars and ethanol were consumed by the yeast culture, a spike in the DO occurred, triggering the algorithm to deliver more syrup into the culture media at a defined feedrate and pulse dose. Thus, repeated pulses increased the fermentation broth volume, which was removed daily from the bioreactor, by reducing the excess volume to 1.1 L. As volume was removed from the bioreactor, a post sterile addition (PSA) solution, containing a known concentration of trace metals, vitamins, and ammonium phosphate monobasic, was added to the culture. Aeration was applied at 1 L/min, pH was controlled at 5.0, by addition of a solution of 25 % ammonium hydroxide, and the temperature was maintained at 30 ºC. The fed-batch fermentation was conducted for 13 days.

2.4 Cell density and total reducing sugars (TRS)

Yeast cell density was analyzed through washed optical density (wOD). The whole cell broth (WCB), aliquoted from the bioreactor, was centrifuged at maximum speed (12300 g) for 5 min in a microtube, the supernatant was removed, the walls of the microtube were cleaned with a cotton swab and the volume was replaced with water. Absorbance was measured at 600 nm in a

spectrophotometer (Shimadzu UV-1900 UV-VIS Spectophotometer) and dilutions were performed to read absorbance values between 0.1 and 0.6.

The concentrations of total reducing sugars (TRS) in the supernatants of fermentation broth were determined by photometry in a ThermoScientific Gallery™ Discrete Analyzer instrument, using the reagent kit 984317, which contains betafructosidase, hexokinase, glucose-6-phosphate isomerase and glucose-6-phosphate dehydrogenase enzymes. Standards containing sucrose, glucose and fructose were used for the construction of calibration curves. The absorbance of samples was measured at a wavelength of 340 nm and temperature of 37 °C, and the results were expressed in g/L.

2.5 β-Farnesene quantification through gas chromatography

β-farnesene was quantified by Gas chromatography (GC) using an Agilent 8890 GC System with a flame ionization detector (FID). The autosampler was the Agilent 7693A Autosampler. Separation column was Phenomenex ZB-5HT Inferno, with 30+5 m guardian, 0.25 mm and 0.25 µm film (reference: 7HG-G015-11-GGA). Sample carrier gas was hydrogen, 1 µL of injection volume was used and the column temperature ranged from 60 to 325 ºC. Farnesene from the WCB was extracted by using a methanol solution. A set of β-farnesene standard dilutions was used for constructing the calibration curves.

Farnesene yield (%) and productivity (g/L/h) were determined according to Equation 1 and 2, respectively. For calculating the yield, the mass of

farnesene (g) produced ($m_{farnesne}$) and the mass of TRS (g) consumed (m_{TRS}) were used. For the determination of productivity, $m_{farnesne}$, the volume of broth (L) inside the bioreactor (V_{broth}) and the incubation time (t, in h) were used.

$$Farnesene\ yield = \frac{m_{farnesene}}{m_{TRS}} \quad (1)$$

$$Farnesene\ productivity = \frac{m_{farnesne} \div V_{broth}}{t} \quad (2)$$

2.6 Weibull kinetic model

The Weibull model was applied for describing mathematically the accumulation kinetics of phenolic compounds during 13-days of β-farnesene fermentation (Equation 3; Weibull (1951)). In this model, *Ct* represented the predicted concentration of phenolic compounds (mg/L) at incubation time equal to *t. C*∞ represented the equilibrium concentration (mg/L) of each phenolic compound along the 13 days of fermentation. *β* represented the scale parameter a behavior index, the exponent *n* indicated the shape of the phenolic accumulation curve and *t* represented the incubation time.

$$\boldsymbol{C_t = C_\infty \times \left(1 - e^{-\beta t^n}\right)} \quad (3)$$

The estimation of the kinetics parameters was carried out by non-linear regression by using Excel (Microsoft Office 365). The adequacy of the kinetic model proposed was evaluated by two goodness-of-fit criteria, namely coefficient of determination (R^2) and root mean square error (RMSE), following Equation 4. C_{iexp} and C_{icalc} were the experimental and calculated phenolic concentration (mg/L), respectively, and n was the number of experimental data

points in each quantification. The results were presented as the mean ± standard deviation of four bioreactor fermentations.

$$RMSE = \sqrt{\frac{\sum_{i=1}^{n}(C_{iexp} - C_{icalc})^2}{n}} \quad (4)$$

2.7 Theoretical mass balance

The theoretical content of phenolic compounds inside the bioreactor ($C_{phenols\ in\ broth}$) was determined by applying a mass balance approach (Equations 5, 6 and 7). This mass balance was performed for the main fermentation times. TPC and the concentration of each identified phenolic compound class were calculated with Equation 5 for each analyzed time, in which $V_{broth\ current}$ represented the volume of culture broth at that time. The mass of phenolic compounds from the previous fermentation time ($m_{phenols\ in\ previous\ broth}$) was calculated with Equation 6, by using the volume of broth that was transferred to the subsequent fermentation time ($V_{broth\ transfered}$) and the concentration of phenolic compounds in the broth of the previous estimation ($C_{phenols\ in\ previous\ broth}$). The mass of phenolic compounds added to the fermentation broth ($m_{phenols\ in\ syrup\ fed}$) was determined according to equation 7, where $C_{phenols\ in\ syrup}$ and $V_{syrup\ fed}$ represented the concentration of phenolic compounds in the syrup and the volume of syrup supplied, respectively.

$$C_{phenols\ in\ broth} = \frac{m_{phenols\ in\ previous\ broth} + m_{phenols\ in\ syrup\ fed}}{V_{broth\ current}} \quad (5)$$

$$m_{phenols\ in\ previous\ broth} = V_{broth\ transfered} \times C_{phenols\ in\ previous\ broth} \quad (6)$$

$$m_{phenols\ in\ syrup\ fed} = V_{syrup\ fed} \times C_{phenols\ in\ syrup} \quad (7)$$

2.8 Statistical analysis

The results obtained were analyzed by using the statistical program STATISTICA version 14.0.0.15 for Windows. Normality of data distribution was tested by Shapiro-Wilk method. The determination of statistical significance was calculated by one-way analysis of variance (ANOVA) with Fisher LSD post hoc test to compare groups' means and student's t-test. The results were considered statistically significant when $p < 0.05$.

3. Results and discussion

3.1 Sugarcane syrup characterization

Two batches of sugarcane syrup were characterized by determining TRS, density, pH and TPC (Table 1). The batches presented significant differences ($p < 0.05$) in all parameters measured. The average of TPC in both batches was 50.66 mg/L, with 17.6 % higher ($p < 0.05$) concentration in batch A than in batch B, corresponding to a difference of 8.22 mg/L. This variation in the properties of the sugarcane syrup may be attributed to the seasonality of the raw material. In fact, the difference in seasons has been found to change the condition of the soil and climate, which are responsible for altering the composition of the sugarcane (Agu & Oduola, 2021). However, since both batches were produced from sugarcane harvested in the beginning of spring in

Brazil (September), the differences found may not be due to the harvesting timing.

Parameter	Batch A	Batch B	Average
TRS (%)	57.68 ± 0.28 (a)	58.66 ± 0.04 (b)	58.17 ± 0.20
Density (g/mL)	1.28 ± 0.00 (a)	1.30 ± 0.00 (b)	1.29 ± 0.01
pH	5.93 ± 0.01 (a)	5.75 ± 0.03 (b)	5.84 ± 0.09
TPC (mg/L)	54.77 ± 0.57 (a)	46.55 ± 0.74 (b)	50.66 ± 4.16
Hydroxybenzoic acids (mg/L)	14.20 ± 0.15 (a)	15.12 ± 0.14 (b)	14.66 ± 0.48
Hydroxycinnamic acids (mg/L)	28.22 ± 0.07 (a)	22.19 ± 0.35 (b)	25.21 ± 3.03
Flavonoids (mg/L)	12.35 ± 0.88 (a)	9.24 ± 0.25 (b)	10.80 ± 1.62

Table 1. Characterization of sugarcane syrup used in fermentations in terms of TRS, density, pH, TPC and each class of phenolic compounds. Different letters in the same row - (a) or (b) - represent the significant differences from a t-test (n = 3) between syrup batches for each parameter.

A detailed characterization of the phenolic compounds identified by LC-ESI-QqTOF-HRMS showed the presence of three chemical classes, including hydroxybenzoic acids, hydroxycinnamic acids and flavonoids (Table 2). The hydroxycinnamic acids were the dominant class of phenolic compounds with 13 different compounds identified, representing 49.6 % of all the compounds quantified. The molecule with the highest concentration identified in both batches of sugarcane syrup was *trans*-3-feruloylquinic acid (m/z 367 $[M-H]^-$ ($C_{17}H_{19}O_9$)), with an average of 7.22 ± 0.15 mg/L. On the other hand, *p*-coumaric acid, isoferulic acid, chlorogenic acid, 5-O-feruloylquinic acid and a caffeoylquinic acid derivative presented lower concentrations, ranging between 1.28 and 3.85 mg/L. The prevalence of these phenolic compounds is in accordance with the literature, where Duarte-Almeida et al. (2011) described sugarcane stalks to contain mainly phenylpropanoids, which englobe compounds such as caffeic, chlorogenic and coumaric acids. Other works also

described the presence of compounds like *trans*-3-feruloylquinic, coumaroylquinic, *trans*-4-caffeoylquinic, chlorogenic, caffeic, coumaric and ferulic acids in sugarcane plants (Barrera et al., 2020; Coutinho et al., 2016; Payet et al., 2006). Chlorogenic acid has previously been detected in sugarcane juices and molasses, and a relation with enzymatic browning was established (Duarte-Almeida et al., 2011). Many of the identified hydroxycinnamic acids have been described to have antimicrobial activity against *S. cerevisiae*: caffeoylquinic acid, with an IC_{80} at 10000 mg/L (Bajko et al., 2015), caffeic acid, with a MIC at 3600 mg/L (Merkl et al., 2010), *p*-coumaric acid, with total inhibition at 1200 mg/L (Adeboye et al., 2015; Gu et al., 2015) and ferulic acid, with 76 % growth inhibition at 1000 mg/L (Gu et al., 2015; Merkl et al., 2010). (Gu et al., 2015; Merkl et al., 2010). On the other hand, all phenolic compounds have also been identified as antioxidant agents, such as feruloylquinic acids, with an ABTS IC_{50} between 4 and 13 mg/L (Yang et al., 2013), caffeoylquinic acids, with DPPH IC_{50} between 24 and 51 µg (Karamać et al., 2012) and coumaroylquinic acids, with DPPH at around 600 mg ascorbic acid equivalent per g (Hammoda et al., 2013).

Proposed compound	Retention time (min)	Molecular Formula -H	*m/z* Measured $[M-H]^-$	MS/MS fragments	Error (ppm)	mSigma (Da)	Sugarcane syrups concentration (mg/L)	
							Batch A	Batch B
Hydroxybenzoic acids								
4-Hydroxybenzaldehyde	8.7	$C_7H_5O_2$	121.0295	121	2.7	3.4	1.41 ± 0.03 (a)	4.47 ± 0.04 (b)
o-Hydroxybenzoic acid	4.7	$C_7H_5O_3$	137.0244	108	1.3	5.4	ND	0.91 ± 0.01
4-Hydroxybenzoic acid	7.2	$C_7H_5O_3$	137.0221	93, 137	2.9	1.7	1.68 ± 0.05	ND
Protocatechuic acid	5.7	$C_7H_5O_4$	153.0193	109, 153	3.2	5.4	0.94 ± 0.01 (a)	0.66 ± 0.02 (b)
2,6-Dihydroxybenzoate	8.5	$C_7H_5O_4$	153.0194	109	1.7	7.2	ND	1.31 ± 0.05
Gentisic acid	9.2	$C_7H_5O_4$	153.0193	65, 109	2.2	2.5	1.73 ± 0.01	ND
Hydroxybenzoic-4-B-glucoside	4.8	$C_{13}H_{15}O_8$	299.0717	108, 152	2.1	3.1	1.64 ± 0.03 (a)	2.66 ± 0.07 (b)
Gentisic acid derivatives	5.4	$C_{13}H_{15}O_9$	315.0722	108, 152	1.7	4.7	5.87 ± 0.04 (a)	4.91 ± 0.09 (b)
Hydroxycinnamic acids								
p-Coumaric acid	10.3	$C_9H_7O_3$	163.0401	119	1.9	8.0	3.85 ± 0.15 (a)	3.05 ± 0.06 (b)
p-Coumaric acid derivative	10.9	$C_9H_7O_3$	163.0401	119	1.9	8.0	0.24 ± 0.02 (a)	0.11 ± 0.00 (b)
Caffeic acid	8.5	$C_9H_8O_4$	179.0317	135, 179	3.0	6.3	0.89 ± 0.01 (a)	0.79 ± 0.03 (a)

Ferulic acid	9.7	$C_{10}H_9O_4$	193.0506	134	2.8	9.3	0.77 ± 0.05 (a)	0.35 ± 0.02 (b)
Isoferulic acid	11.2	$C_{10}H_9O_4$	193.0479	134, 161, 193	3.5	5.8	2.19 ± 0.08 (a)	1.50 ± 0.05 (b)
4-*p*-Coumaroylquinic acid	7.4	$C_{16}H_{17}O_8$	337.0929	119, 163	1.9	2.6	0.68 ± 0.01 (a)	0.54 ± 0.02 (b)
p-Coumaroylquinic acid	9.2	$C_{16}H_{17}O_8$	337.0929	93, 163, 173, 191	2.1	2.5	1.54 ± 0.01 (a)	1.63 ± 0.05 (a)
Neochlorogenic acid	6.3	$C_{16}H_{17}O_9$	353.0878	135, 179, 191	1.6	4.1	1.59 ± 0.03 (a)	0.47 ± 0.03 (b)
Chlorogenic acid	7.8	$C_{16}H_{17}O_9$	353.0878	191	1.7	3.2	2.14 ±0.01 (a)	1.99 ± 0.05 (a)
4-Caffeoylquinic acid	8.0	$C_{16}H_{17}O_9$	353.0878	135, 173, 179, 191	2.2	2.0	1.10 ± 0.00 (a)	0.41 ± 0.00 (b)
5-O-Feruloylquinic acid	8.1	$C_{17}H_{19}O_9$	367.1035	134, 193	0.0	ND	3.49 ± 0.02 (a)	3.01 ± 0.07 (b)
trans-3-Feruloylquinic acid	9.8	$C_{17}H_{19}O_9$	367.0596	173	1.6	2.7	7.09 ± 0.11 (a)	7.36 ± 0.00 (a)
caffeoylquinic acid	9.9	$C_{25}H_{24}O_{12}$	515.1195	515	3.2	7.3	2.66 ± 0.04 (a)	1.28 ± 0.00 (b)
Flavonoids								
Tricin/ 3',5'-O-Dimethyltricetin	17.7	$C_{17}H_{13}O_7$	329.0667	299	2.2	1.2	0.14 ± 0.01	ND
Vitexin/Apigenin-8-C-glucoside derivative a	8.6	$C_{21}H_{19}O_{10}$	431.0984	89, 179	1.7	8.5	ND	0.02 ± 0.02
Vitexin/Apigenin-8-C-glucoside derivative b	9	$C_{21}H_{19}O_{10}$	431.0984	311, 341, 431	0.8	10.0	1.09 ± 0.03 (a)	0.89 ± 0.04 (b)
Vitexin/Apigenin-8-C-glucoside c	11.2	$C_{21}H_{19}O_{10}$	431.0984	311, 341	2.8	18.9	0.27 ± 0.01 (a)	0.12 ± 0.00 (b)
Vitexin/Apigenin-8-C-glucoside d	11.4	$C_{21}H_{19}O_{10}$	431.0984	311, 341	2.4	8.1	ND	0.14 ± 0.00
Isoorientin/ Luteolin-8-C-glucoside	10.3	$C_{21}H_{19}O_{11}$	447.0933	327, 357	3.9	14.3	0.12 ± 0.01	ND
Orientin/Luteolin-8-C-glucoside 1	10.4	$C_{21}H_{19}O_{11}$	447.0933	327, 357	3.5	10.8	0.07 ± 0.00	ND
6-Methoxyluteolin 7-rhamnoside derivative a	10.5	$C_{22}H_{21}O_{11}$	461.1089	461	3.1	6.1	0.12 ± 0.00 (a)	0.11 ± 0.01 (a)
6-Methoxyluteolin 7-rhamnoside derivative b	11.8	$C_{22}H_{21}O_{11}$	461.1089	341, 371	4.4	12.8	0.16 ± 0.00	ND
tricin-7-O-glucoside	12.4	$C_{25}H_{31}O_{10}$	491.1826	329	4.2	13.5	0.94 ± 0.02 (a)	0.55 ± 0.02 (b)
Isovitexin 2"-O-arabinoside	9.5	$C_{26}H_{27}O_{14}$	563.1406	353, 443	2.9	5.3	0.24 ± 0.01 (a)	0.06 ± 0.01 (b)
Isoschaftoside	10.1	$C_{26}H_{27}O_{14}$	563.1406	353, 473	1.9	3.3	5.33 ± 0.05 (a)	4.21 ± 0.07 (b)
Neoschaftoside	10.5	$C_{26}H_{27}O_{14}$	563.1406	399, 473	3.2	10.4	0.81 ± 0.07 (a)	0.44 ± 0.04 (b)
Apigenin 7-O-neohesperidoside	11.3	$C_{27}H_{29}O_{14}$	577.1563	293, 413	1.3	6.9	0.22 ± 0.03 (a)	0.05 ± 0.00 (b)
Tricin-O-neohesperoside isomer	13.4	$C_{29}H_{33}O_{16}$	637.1638	329	1.1	11.5	0.27 ± 0.00 (a)	0.12 ± 0.00 (b)
Tricin-7-O-rhamnosyl-glucuronide	13.1	$C_{29}H_{31}O_{17}$	651.1567	329	2.9	4.7	0.83 ± 1.06 (a)	1.50 ± 0.01 (a)
Tricin diglucuroniside	11.5	$C_{29}H_{29}O_{19}$	681.1322	351	1.0	15.7	1.43 ± 0.28 (a)	0.90 ± 0.03 (a)
tricin-4-(O-erythro) ether glucoside	14.5	$C_{33}H_{35}O_{16}$	687.1786	195, 329, 491, 525	1.5	6.3	0.05 ± 0.00	ND

Table 2. LC-ESI-UHR-QqTOF-MS results of phenolic compounds detected in sugarcane syrup. ND – Not detected. Different letters in the same row - (a) or (b) - represent the significant differences from a t-test (n = 2) between syrup batches.

The second most represented class of phenolic compounds in the sugarcane syrup was the class of hydroxybenzoic acids, with a mean prevalence of 29.2 % in both batches analyzed. Eight different compounds within this class were identified (Table 2). Among them, 4-hydroxybenzaldehyde and hydroxybenzoic-4-β-glucoside were detected by the presence of the ions m/z 121 $[M-H]^-$ ($C_7H_5O_2$) and m/z 299 $[M-H]^-$ ($C_{13}H_{15}O_8$). In addition, two peaks

with a m/z 315 $[M-H]^-$ ($C_{13}H_{15}O_9$) were identified as gentisic acid-2-O-β-glucoside and gentisic acid-5-O-β-glucoside and were described as gentisic acid derivatives. Gentisic acid derivatives were found to be the predominant hydroxybenzoic acid (5.39 ± 0.48 mg/L), followed by 4-hydroxybenzaldehyde (2.94 ± 1.53 mg/L), and hydroxybenzoic-4-β-glucoside (2.15 ± 0.51 mg/L). Many hydroxybenzoic acids have been previously found in the sugarcane plant, including protocatechuic, *p*-hydroxybenzoic and gentisic acids, and hydroxybenzoic-4-β-glucoside (Coutinho et al., 2016; Payet et al., 2006). Hydroxybenzaldehyde and protocatechuic acid have been reported to have both antioxidant and antimicrobial activities on *S. cerevisiae*, with growth inhibition at 300 and 3100 mg/L, respectively (Farhoosh et al., 2016; Gu et al., 2015; Merkl et al., 2010; Nobsathian et al., 2017). Merkl et al. (2010) have also reported antimicrobial activity of 4-hydroxybenzoic acid at 690 mg/L.

Another class of phenolic compounds identified in the syrup was flavonoids, in which 18 different peaks were recognized in LC-ESI-QqTOF-HRMS (Table 2) and represented 21.2 % of TPC. This class was represented by isoschaftoside (m/z 563 $[M-H]^-$ ($C_{26}H_{27}O_{14}$), which was the second most concentrated phenolic compound with a concentration of 4.77 ± 0.56 mg/L, and has been linked to antioxidant activity (Vila et al., 2008). Apigenin, isoschaftoside and neoschaftoside (apigenin derivatives), tricin-O-neohesperoside isomer, tricin-7-O-rhamnosyl-glucuronide and tricin-4-(O-erythro) ether glucoside were previously detected in sugarcane (Coutinho et al., 2016; Deseo et al., 2020; Duarte-Almeida et al., 2006; Eggleston, 2018). Some specific compounds, namely flavonoids with C-glucoside groups, have been

associated with antioxidant and antimicrobial activities, and the plants use them to attract insects and promote mycorrhizal symbioses (McNally et al., 2003). Flavonoids with C-glucoside groups are important compounds for defense and if they are in the plant during syrup extraction season, they will be present in sugarcane syrups. Other identified molecules, namely tricin, apigenin and luteolin, have been associated to antimicrobial properties, through decreasing membrane fluidity (Tsuchiya, 2015; Zhou & Ibrahim, 2010). Most of the identified flavonoids have been described to have antioxidant activity, namely tricin (Shang et al., 2015); luteolin, with a DPPH IC_{50} at 90 mg/L (Wang et al., 2014); vitexin and isovitexin (apigenin C-glycosides isomers), with ABTS ascorbic acid equivalents around 80 and 8 mg/L, respectively (Khole et al., 2016).

Overall, the phenolic composition of sugarcane syrup was in accordance with previous works that describe the composition of the sugarcane plant, namely the leaves, stalk, juice and molasses. However, these molecules are associated with different effects on microorganisms, providing oxidative stress reduction, by exerting antioxidant activity, or/and inhibiting cellular growth, by exerting antimicrobial activity. These effects are dependent on the phenolic concentration and fermentation conditions (Adeboye et al., 2015; Adeboye et al., 2014).

3.2 Fermentation profile

Fed-batch fermentations in 2 L bench bioreactors were conducted by simulating the industrial Amyris fermentation process of β-farnesene production. During the fermentation, several parameters were measured, including cell density, TRS, syrup addition and farnesene concentration. The results are presented as the average of 4 independent bioreactor fermentations.

Cell density, which was measured through wOD (Figure 1A), increased during the first three days of cultivation from 18.5 to 128.5, reaching a stationary phase on day 3 and then varying between about 130 and 150 until the end of the fermentation. After the 3rd day, high cell density was achieved, which is characteristic in fed-batch fermentations, and is one of the requirements to achieve a high product concentration and productivity (Subramaniam et al., 2018). The initial TRS concentration inside the fermentation broth (Figure 1A) was 22.02 g/L, then it decreased to about 1 g/L at the end of 24 hours and finally remained at residual concentrations during the rest of the process. The presence of residual amounts of TRS implies that all sugars supplied through the sugarcane syrup were consumed (Figure 1B), leading to no accumulation inside the bioreactors. Therefore, the algorithm used for feedstock addition was robust enough to maintain the concentration of sugars low, minimizing the Crabtree effect and reducing the formation of by-products (such as ethanol). Consequently, this allows to achieve higher product yield during the fermentation (Meadows et al., 2016).

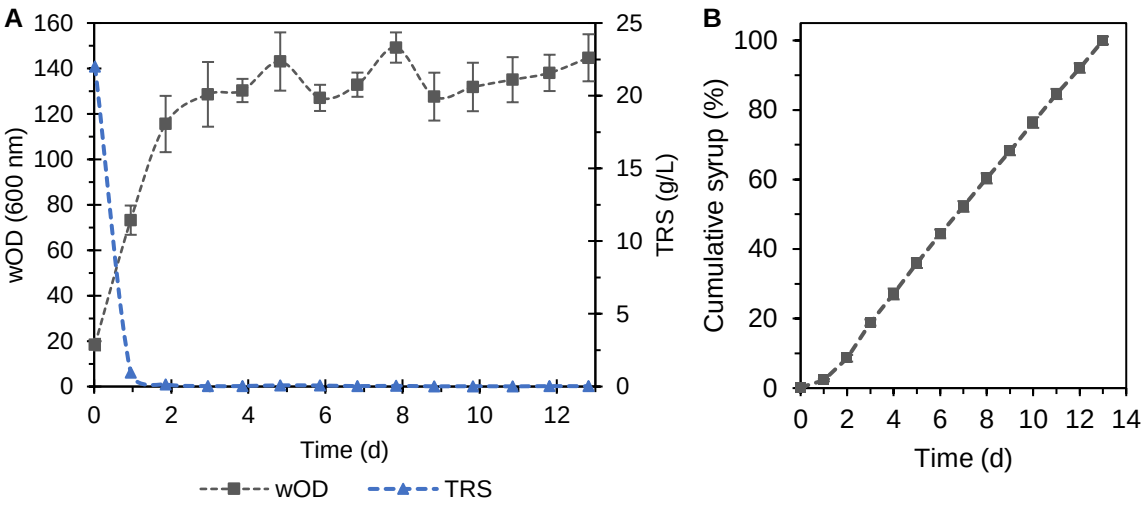

Figure 1. Washed optical density measured at 600 nm and total reducing sugars (TRS) (A), and normalized cumulative sugarcane syrup (B) through 13 days of fed-batch fermentations of farnesene producing *S. cerevisiae*. Results represent the average of 4 bioreactor fermentations and error bars are standard deviations.

In all bioreactor fermentations performed in this work, the initial culture medium for the main fermentation was supplied with a pulse of syrup providing sugars to the yeast. The culture medium corresponded to 98.3 % of broth weight and the syrup to 1.7 % (Figure 2). The mass of syrup added into the bioreactors increased over time (Figure 1B). Apart from sugarcane syrup, the bioreactor was also fed with an ammonium hydroxide solution to adjust the pH and with the PSA solution to supply micronutrients to the yeast. Both solutions did not contribute to the weight of the broth in a significant way, with a prevalence of only 2.1 % for the ammonium hydroxide solution and 0.3 % for PSA. On the other hand, it was possible to observe the increase of syrup proportion inside the bioreactor over time, reaching 86.8 % in the end of the process. Considering this, molecules that are provided from the syrup, such as the sugars, that were consumed, did not accumulate inside the culture medium

(Figure 1A). In contrast, any substance (such as phenolic compounds) that was supplied with the syrup and was not degraded or metabolized by the yeast could continuously accumulate inside the broth.

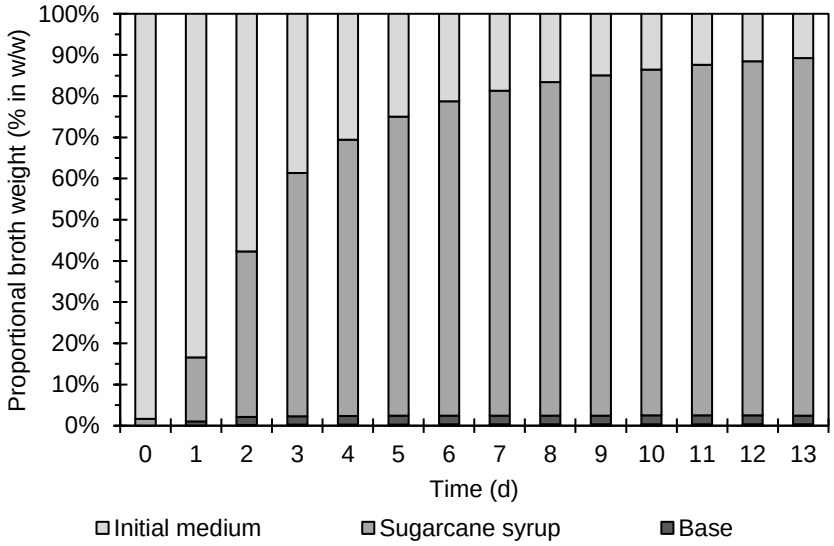

Figure 2. Proportional broth weight (%) of all medium components (initial medium, sugarcane syrup and base) during 13 days of fed-batch fermentations of farnesene producing *S. cerevisiae*. Results represent the average of 4 bioreactor fermentations and error bars are standard deviations.

The concentration, yield and productivity of farnesene was determined over the fermentation time to evaluate the performance of farnesene production from the sugarcane syrup batches used. The yield of farnesene ($g_{farnesene}/g_{sugar}$) presented a quite constant profile, with values above 90% of the yield obtained in the last day, between day 1 and day 5, and above 97%, after day 6 of fermentation (Figure 3). The concentration of farnesene (g/L), and the farnesene cumulative productivity (g/L/h), increased rapidly during the first days of the process, stabilizing after 6/7 days of fermentation. Namely, the productivity increased from 30% to more than 90% of the productivity of the last day, from day 1 to day 5, staying above 95% of maximum values from day 6 to

day 13 of fermentation (Figure 3). Furthermore, variation in farnesene parameters was below 5 % between fermentations. Thus, it can be concluded that the recombinant biomolecule was stably produced in the broth of the high cell density system generated by fed-batch mode, regardless of the syrup batch used.

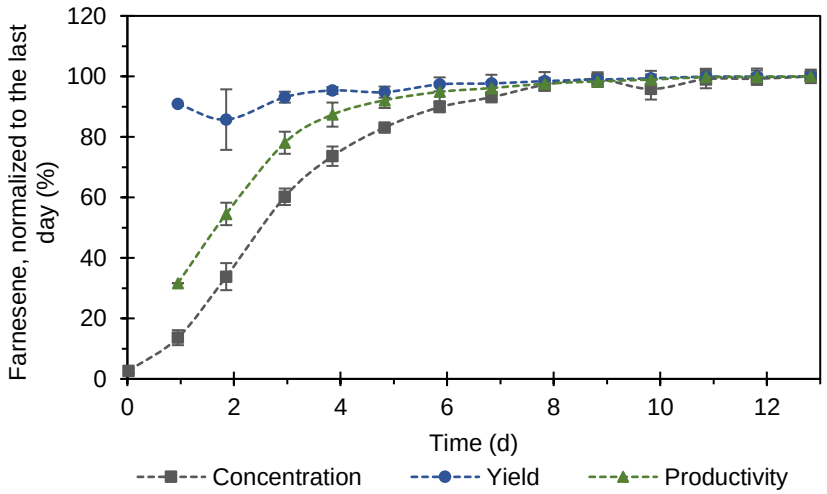

Figure 3. Farnesene concentration (g farnesene/L broth), yield (g farnesene/ g TRS) and productivity (g farnesene/L broth/ h) normalized in relation to the last day of fermentation during 13 days of fed-batch fermentations of farnesene producing *S. cerevisiae*. Results represent the average of 4 bioreactor fermentations and error bars are standard deviations.

3.3 Phenolic compounds during fed-batch fermentation

The concentrations of the phenolic compounds identified during the 13 days of fed-batch fermentations are presented in Figure 4. The lowest concentration of most identified phenolic compounds was detected in the beginning of the fermentation, starting to increase over time until day 3 and reaching a stable range of values. This was the evolution profile obtained for the

most representative compounds of each class, such as gentisic acid, feruloylquinic acid and schaftoside. An increasing phenolic profile was expected because they were added semi-continuously together with the syrup.

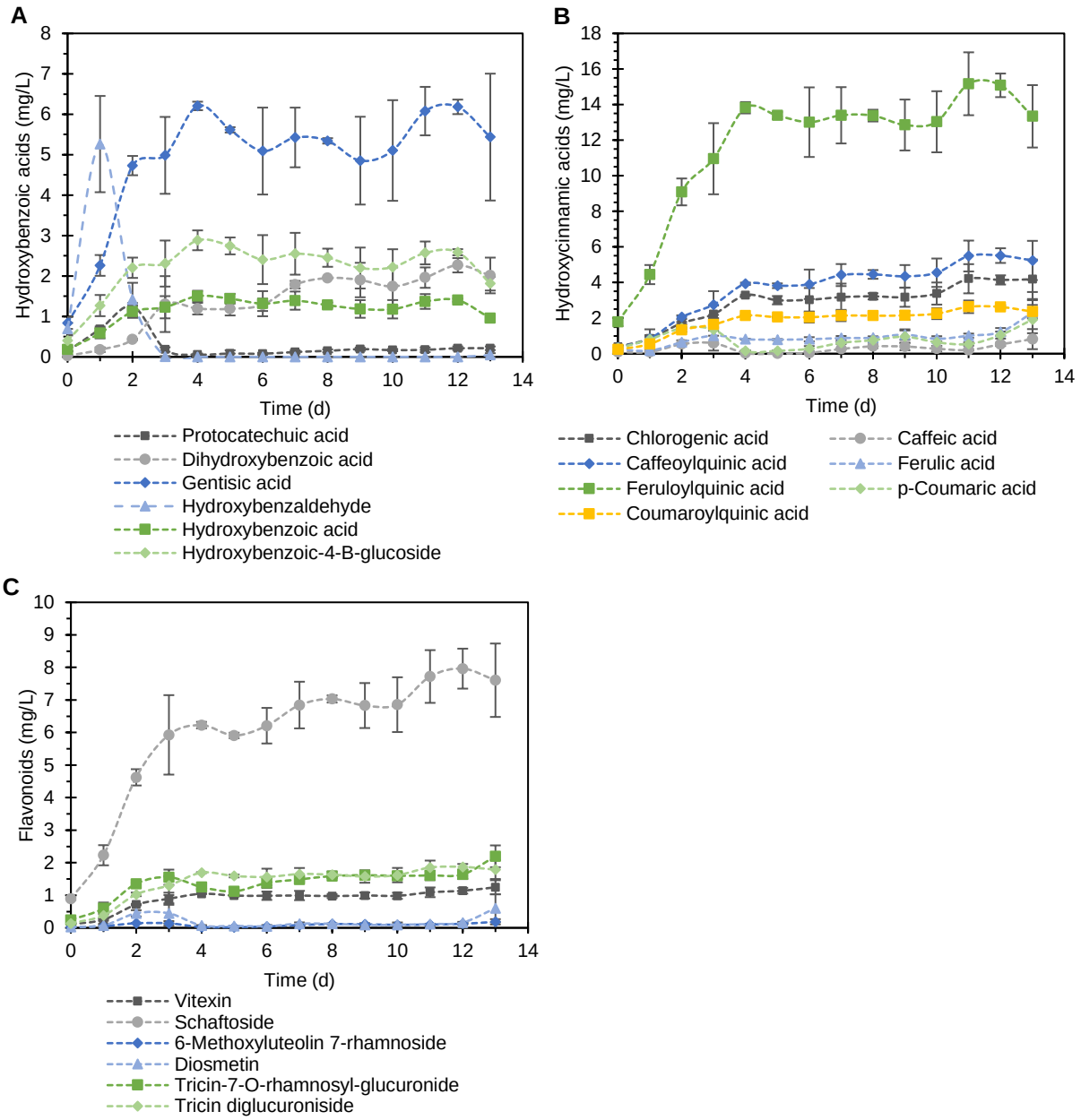

Figure 4. Identified phenolic compounds measured in LC-ESI-QqTOF-HRMS during 13 days of fed-batch fermentations of farnesene producing *S. cerevisiae*. A –

hydroxybenzoic acids, B – hydroxycinnamic acids, C – flavonoids. Results represent the average of 4 bioreactor fermentations and error bars are standard deviations.

However, for some molecules, such as hydroxybenzaldehyde and protocatechuic acid (two hydroxybenzoic acids), caffeic, ferulic and *p*-coumaric acids (three hydroxycinnamic acids) and diosmetin (a flavonoid), a decrease of concentration was observed from day 2 until the end of fermentation. Metabolization (or degradation) of hydroxybenzaldehyde, ferulic and *p*-coumaric acids by *S. cerevisiae* have been previously reported (Fletcher et al., 2019; Gu et al., 2015; Jönsson et al., 1998; Richard et al., 2015). The decrease in concentration of these compounds observed from day 2 of the fermentation can be explained with yeast metabolization. These molecules have high antimicrobial activity (Gu et al., 2015; Merkl et al., 2010). Therefore, it could be beneficial for the yeast to metabolize these compounds into other less toxic molecules. In fact, hydroxybenzoic acid derivatives might have been converted into hydroxybenzoic-4-β-glucoside and caffeic acid into caffeoylquinic acid, which have lower antimicrobial activities (Fletcher et al., 2019; Gu et al., 2015). This would corroborate the increased concentrations ($p < 0.05$) of hydroxybenzoic-4-β-glucoside and caffeoylquinic acid in the end of the fermentation compared to their concentration in the syrup, at 1.47 and 1.93-fold, respectively.

The results of TPC in Figure 5 are the sum of the phenolic concentrations from each phenolic compound class, quantified in each fermentation time point. By the end of the fermentation, TPC was 53.87 ± 13.79 mg/L, with

hydroxybenzoic acids present at 11.43 ± 2.47 mg/L, hydroxycinnamic at 27.93 ± 7.13 mg/L and flavonoids at 14.51 ± 4.07 mg/L. These concentrations are not significantly different ($p > 0.05$) from those detected in the syrup (Table 1). This means that, in the β-farnesene fermentation process, phenolic compounds are accumulating to the levels found in the feedstock.

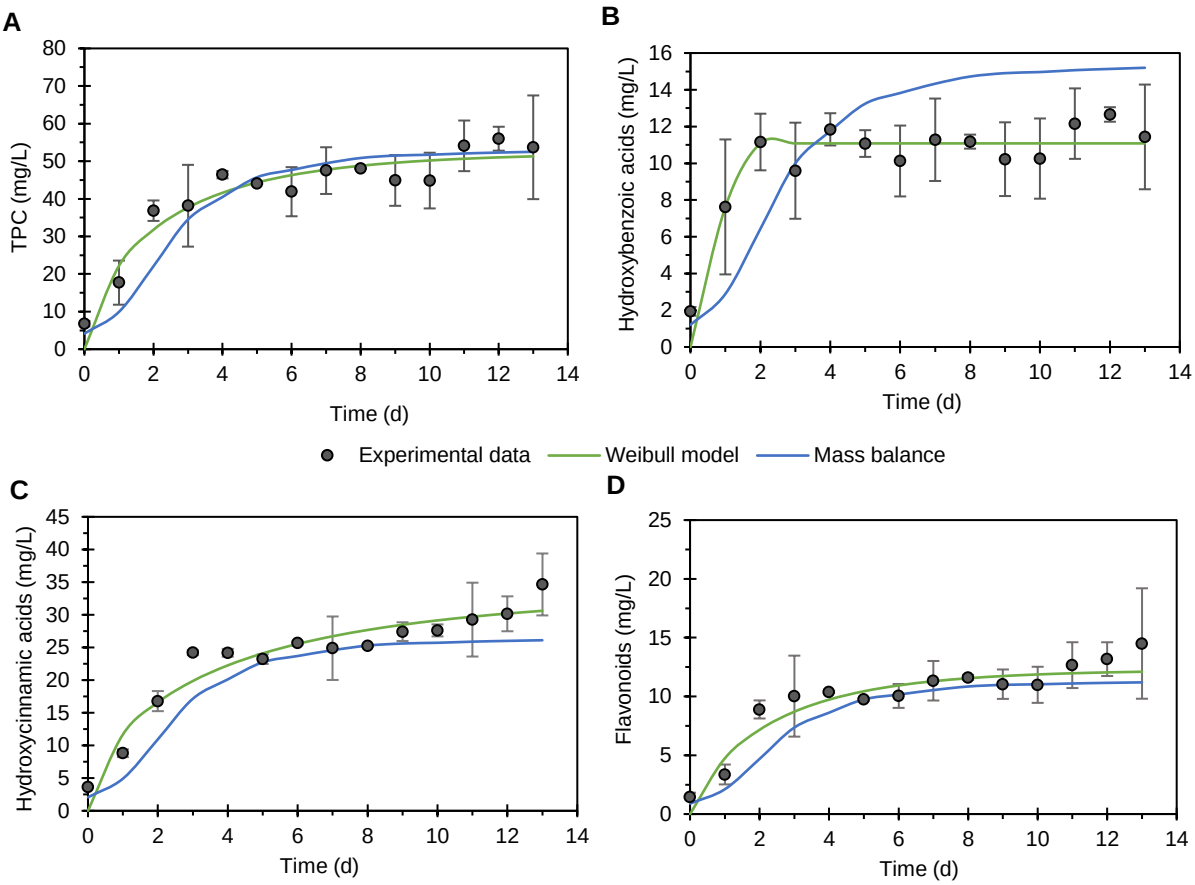

Figure 5. Total phenolic content (TPC) (A), hydroxybenzoic acids (B), hydroxycinnamic acids (C) and flavonoids (D) measured in LC-ESI-UHR-QqTOF-MS during 13 days of fed-batch fermentations of farnesene producing *S. cerevisiae*. Plotted dots represent the data for the average of 4 reactor fermentations, error bars are standard deviations and plotted lines correspond to the values estimated from the fitted Weibull model and the theoretical mass balance.

3.4 Predictive modulation for phenolic accumulation in fed-batch fermentation

In order to describe the transfer of phenolic compounds from the sugarcane syrup into the bioreactors during the fed-batch fermentations, two calculations were performed. The Weibull kinetic model was applied to the phenolic experimental data obtained along the 13-days of fermentation (Table 3). This model presented a good adjustment to the experimental data during the fermentation time (Figure 5). In TPC and all the classes identified, the kinetic parameter that represents concentration at equilibrium (C_∞) presented no significant differences with the concentration of phenolic compounds detected in the syrup – using a different batch of syrup. The values of *n* of the power law model showed that the diffusion of some compounds (with $n > 1$) was controlled by a Fickian behavior where the compounds absorption increased linearly as a function of the time, then reaching a plateau region and finally the curve became constant. On the other hand, values of $n < 1$ for the Weibull model described the parabolic shape of the curves with a high initial slope. Regarding the β parameter, it can be considered as the constant accumulation rate for phenolic compounds inside the bioreactors and the higher values were associated with the fastest accumulation kinetics (Alonso-Riaño et al., 2020).

Phenolic content	C_∞ (mg/L)	β	n	R^2	RMSE
TPC	46.59 ± 9.59 ^{A}	0.46 ± 0.17 ^{B}	0.88 ± 0.28 ^{AB}	0.93	1.16
Hydroxybenzoic acids	12.40 ± 4.85 ^{C}	0.97 ± 0.46 ^{A}	0.83 ± 0.64 ^{B}	0.93	0.20
Hydroxycinnamic acids	31.36 ± 13.12 ^{B}	0.33 ± 0.09 ^{B}	0.82 ± 0.28 ^{B}	0.94	0.64
Flavonoids	10.98 ± 0.83 ^{C}	0.36 ± 0.06 ^{B}	1.60 ± 0.56 ^{A}	0.85	0.26
Dihydroxybenzoic acid	2.08 ± 1.02 ^{d}	0.09 ± 0.07 ^{d}	1.62 ± 0.83 ^{a}	0.92	0.05
Gentisic acid	4.68 ± 1.37 ^{c}	0.68 ± 0.19 ^{a}	1.87 ± 0.49 ^{a}	0.92	0.17
Hydroxybenzoic-4-β-glucoside	2.76 ± 0.01 ^{d}	0.80 ± 0.05 ^{a}	0.85 ± 0.26 ^{a}	0.88	0.05
Chlorogenic acid	5.39 ± 2.99 ^{bc}	0.21 ± 0.10 ^{cd}	0.73 ± 0.25 ^{a}	0.92	0.10
Caffeoylquinic acid	5.68 ± 1.63 ^{bc}	0.18 ± 0.05 ^{cd}	0.97 ± 0.22 ^{a}	0.96	0.11

Feruloylquinic acid	12.83 ± 1.59 [a]	0.40 ± 0.03 [b]	1.22 ± 0.31 [a]	0.96	0.30
Coumaroylquinic acid	2.34 ± 0.30 [d]	0.29 ± 0.07 [bc]	1.09 ± 0.25 [a]	0.96	0.07
Vitexin	1.22 ± 0.29 [d]	0.32 ± 0.11 [bc]	1.14 ± 0.73 [a]	0.91	0.02
Schaftoside	7.18 ± 1.43 [b]	0.42 ± 0.07 [b]	0.90 ± 0.19 [a]	0.95	0.14
Tricin-7-O-rhamnosyl-glucuronide	1.63 ± 0.07 [d]	0.43 ± 0.20 [bc]	1.63 ± 0.31 [a]	0.80	0.06
Tricin diglucuroniside	1.77 ± 0.01 [d]	0.29 ±0.06 [c]	1.62 ± 0.19 [a]	0.96	0.04

Table 3. Parameters for Weibull model fitting to phenolic compound experimental data. Estimates (± standard deviation) of Weibull model parameters used to describe TPC, total hydroxybenzoic acids, hydroxycinnamic acids and flavonoids, as well the most predominant within each class, through the incubation time of fed-batch fermentation using sugarcane syrup as feedstock. C∞, β and n: kinetic parameters of the Weibull model. R^2: coefficient of determination. RMSE: root mean square error. Difference in letters in superscript for each model parameter represents significant differences from an ANOVA LSD post-hoc test (n = 2) between total phenolics and phenolic classes (A) and each individual phenolic compound (a).

Additionally, the R^2 for the fitting to the TPC data was 0.93, and this was similar for hydroxybenzoic and hydroxycinnamic acids. The fit to the flavonoids presented a lower R^2, at 0.85. Therefore, the Weibull model was successful at describing the evolution of the most predominant molecules. This model has been previously used in other works, such as in modelling organism inactivation (van Boekel, 2002) and phenol extraction kinetics (Alonso-Riaño et al., 2020). It has also been applied to study fermentation parameters, such as sugar consumption and product formation, in batch lactic acid fermentation (Germec et al., 2018). However, it has never been used to describe the accumulation of molecules, such as phenolic compounds, in a fed-batch process.

The theoretical concentration of phenolic compounds inside the bioreactors was done by a mass balance of entries of syrup and removals of broth over time, using the concentration of phenolic compounds measured in

544 the syrup (Table 4). No significant differences have been found between both
545 batches of syrup. The fit of this calculation presented a similar profile to the
546 experimental data, with an increase of phenolic compounds over the
547 fermentation time (Figure 5). Regarding the fit to data of TPC, hydroxycinnamic
548 acids and flavonoids, it presented an R^2 of 0.86 or higher. However, the poorest
549 adjustment to the experimental data was obtained for the hydroxybenzoic acids
550 class, with an R^2 of 0.63. This result can be explained by the fact that the mass
551 balance does not consider any degradation of the molecules, which was clear in
552 this phenolic class. The decrease of the concentration of certain phenolic
553 compounds during the fermentation revealed that the evolution of phenolic
554 compounds in the studied fed-batch fermentation does not just follow a mass
555 balance profile, with the concentration being augmented as syrup is added to
556 the bioreactor, but that the conversion of molecules by the yeast must be also
557 taken into account.

Phenolic compound class	R^2	RMSE
TPC	0.88	1.54
Hydroxybenzoic acids	0.63	0.94
Hydroxycinnamic acids	0.91	0.72
Flavonoids	0.86	0.50

558 Table 4. Coefficient of determination and root mean square error for theoretical mass
559 balance fitting to phenolic compounds (total phenolic content (TPC), total
560 hydroxybenzoic acids, hydroxycinnamic acids and flavonoids) during the fed-batch
561 fermentation using sugarcane syrup as feedstock.

562

563 When comparing the adjustments of both mathematical calculations, the
564 Weibull model was found to present a better fit to the experimental data. It is
565 then possible to conclude that the Weibull model was more effective in

566 describing the evolution of phenolic compounds inside bioreactors during β-
567 farnesene fed-batch fermentations, and that this model may be applied for
568 estimating the levels of these molecules in industrial fermentations.

569

570 ## 4. Conclusions

571 The concentration of most phenolic compounds increased over the 13-
572 days of β-farnesene fermentation until stabilizing after day 3. However, some
573 phenolic compounds decreased after day 2, suggesting their metabolization by
574 the yeast. The two mathematical calculations applied to modulate the phenolic
575 profile, a theoretical mass balance and the Weibull kinetic model, presented
576 good adjustments to data of total phenolic content and each phenolic class.
577 However, the Weibull model presented the better fit, with an $R^2 \geq 0.85$, and thus
578 may be applied as a practical tool to predict the phenolic compound evolution in
579 other fed-batch fermentations using syrup.

580

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