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Carnosic Acid Production from Sugarcane Syrup by Engineered Yeast in Fed-Batch Fermentation

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Abstract: Phenolic diterpene carnosic acid (CA) is widely used in the food, nutritional health, and cosmetic industries due to its antioxidative and antimicrobial properties. This work aimed to overproduce CA in *Saccharomyces cerevisiae* from sugarcane syrup in fed-batch 2 L bioreactor fermentation. A geranylgeranyl diphosphate (GGPP)-producing strain modified with genes encoding the enzymes copalyl diphosphate synthase (Pv.CPS), miltiradiene synthase (Ro.KSL2), hydroxy ferruginol synthase (Ro.HFS), CA synthase (Ro.CYP76AK8), CYP reductase (At.ATR1), and transketolase (TKL1) was used. Lowering the feed rate from 12–26 g/L/h to 7–8 g/L/h, and the use of a dynamic dissolved oxygen (DO) trigger (min. 10%, max. 40%, threshold 70%) instead of a DO trigger of 30%, enhanced CA production by 27%. As a result, the highest CA titer ever reported to date, 191.4 mg/L, was obtained in 4-day fermentation. This study shows the feasibility of engineered yeast to produce CA from the sustainable feedstock sugarcane syrup.

Keywords: carnosic acid; engineered *Saccharomyces cerevisiae*; sugarcane syrup; fed-batch fermentation; dynamic dissolved oxygen trigger

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1. Introduction

Carnosic acid (CA) is a phenolic tricyclic diterpene ($C_{20}H_{28}O_4$) belonging to the large plant secondary metabolite terpenoid class, also known as isoprenoids or terpenes [1]. It has been shown to be a potent antioxidant, having been approved as food additive in several countries [2]. CA also presents other diverse biological properties, such as, anticancer, anti-inflammatory, antimicrobial, antidiabetic, antiobesity and anti-neurodegenerative activities [3]. CA was first discovered, isolated, and characterized from the plant *Salvia officinalis* (common sage) in 1964, and later, at a much higher level (3% *w*/*w* on air dried leaves), in *Rosmarinus officinalis* L. (rosemary) leaves [2]. Rosemary extracts containing CA and its derivative carnosol have since been increasingly used as food preservatives to increase the shelf-life of a wide range of products [3]. However, the amount of CA in plant tissues is not abundant; potential applications are further hampered by an inefficient and resource-demanding extraction process [4]. In addition, the many chemicals in rosemary extract impart a distinctive smell and taste that is not always welcome in an antioxidant additive for food applications. CA and its derivatives also represent a challenging task for chemical synthesis due to their structure, with a more promising approach coming from biotechnological methods, namely yeast fermentation.

The biosynthetic pathway of CA has been elucidated in recent years and its recombinant expression in yeast has been demonstrated [3–7]. CA biosynthesis starts from

geranylgeranyl diphosphate (GGPP), which is derived from isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP) produced via the mevalonate (MVA) pathway or the methylerythritol phosphate (MEP) pathway. It is then cyclized through the action of class II and class I diterpene synthases (CPPS and MiS) to the basic labdane-type terpene skeleton of miltiradiene, which is spontaneously oxidized to abietatriene [5]. The final steps of CA biosynthesis are carried out by cytochrome P450 monooxygenases (CYPs), which catalyze the synthesis of ferruginol by the oxidation of abietatriene at C-12 (CYP76AH22, promiscuous activity) and subsequently add a second hydroxyl group at the position C-11 of ferruginol to produce 11-hydroxyferruginol (CYP76AH22, promiscuous activity), being afterwards oxidized by another cytochrome P450 (CYP76AK8) to produce CA [6]. P450 enzymes require binding to a heme cofactor, and interaction with at least one auxiliary protein (CPR, or cytochrome P450 reductase) that transfers electrons from NADPH to the P450 to help drive its catalytic cycle. Gene expression can also play a major role in this process, as optimal pathway flux balance can help in the mitigation of toxic intermediate accumulation and in the efficiency of enzyme activity, such as promiscuous CYPs. Figure 1 illustrates a schematic diagram of the CA production pathway.

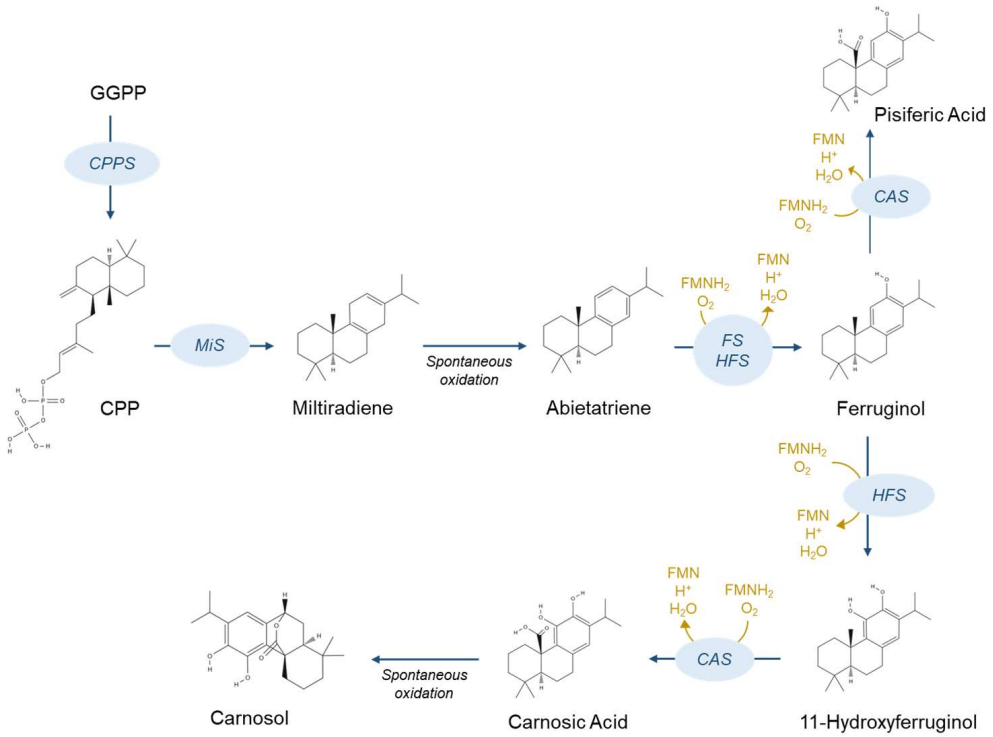


Figure 1. Biosynthetic pathway of CA. Legend: GGPP—geranylgeranyl diphosphate; CPP—copalyl diphosphate; CPPS—copalyl diphosphate synthase; MiS—miltiradiene synthase; FS—ferruginol synthase; CAS—carnosic acid synthase; HFS—hydroxyferruginol synthase. Adapted from [2,3,6].

Few studies report the production of CA by genetically engineered yeast *S. cerevisiae*. In shake flask fermentations, the production levels of CA ranged between 1.00 and 24.65 mg/L [3,5–7]. In 5 L fed-batch fermentation, a CA titer of 75.18 mg/L was obtained, using glucose and ethanol as carbon sources [7]. The large-scale production of value-added molecules through fermentation requires the use of feedstocks, instead of pure sugars, for economic and sustainability reasons. Thus, this work aimed to produce CA by yeast fermentation from sugarcane syrup, a sustainable feedstock with demonstrated efficiency in industrial fermentation [8], using 2 L bioreactors operated in fed-batch mode.

2. Materials and Methods

2.1. Strain

The *Saccharomyces cerevisiae* strain used in this work was obtained by the integration of 6 genes encoding synthases and cofactors for the CA biosynthetic pathway, expressed under the control of pGAL promotors, into the genome of a GGPP-producing strain, proprietary of the Biotechnology Company Amyris Inc. USA (Emeryville, CA, USA): *Talaromyces verruculosus* (*Penicillium verruculosum*) copalyl diphosphate synthase (Pv.CPS) (Uniprot: A0A348FUE1), the *Rosmarinus officinalis* enzyme for the conversion of copalyl diphosphate to miltiradiene (Ro.KSL2) (Uniprot: W8QEG7), *R. officinalis* hydroxyferruginol synthase (Ro.HFS) (Uniprot: A0A0C5Q4Y6), the *R. officinalis* enzyme for the conversion of 11-hydroxyferruginol to CA (Ro.CYP76AK8) (Uniprot: A0A1D8QMG4), cytochrome P450 monooxygenase reduction (At.ATR1) (Uniprot: Q9SB48), and transketolase to improve NADPH cofactor (TKL1) (Uniprot: N1NXM0).

2.2. Fermentations

Small-scale (shake flask) fermentations were performed as described in Lopes et al., 2023 [9]. Fed-batch fermentations were performed in bioreactors of 2.7 L of working volume as described by Carvalho et al., 2022 [10]. The sugarcane syrup used in this work was a Brazilian batch, provided by Amyris Inc. USA (which also has a presence in Brazil), the composition of which was characterized in Carvalho et al., 2024 [11]. Sugarcane syrup pulses were triggered by dissolved oxygen (DO) spikes, which appeared with sugars and ethanol consumption. A DO trigger of 30% and a dynamic DO trigger were tested. The dynamic DO trigger was set as DOTrigger = (((DO_TriggerMin − DO_TriggerMax)/DO_TriggerThreshold) * DOmin) + DO_TriggerMax), with a minimum DO trigger of 10%, maximum DO trigger 40%, and DO trigger threshold 70%. Pulses (10 g of total recoverable sugars (TRS) per liter) of sugarcane syrup (690 g TRS/L of syrup) were delivered at different feed rates (7–8 and 12–26 g/L/h). Fermentations were conducted in duplicate for 4 days. The washed optical density (wOD) was determined spectrophotometrically at 600 nm and the TRS and ethanol photometrically in a ThermoScientific Gallery™ Discrete Analyzer instrument [9,10]. Cell viability was determined through flow cytometry following the methodology of Lopes et al., 2023 [10]. CA, carnosol, ferruginol and pisiferic acid were analyzed by liquid chromatography–electrospray ionization–ultrahigh-resolution–quadrupole time of flight–mass spectrometry (LC-ESI-UHR-QqTOF-MS) (Section 2.4).

2.3. Extraction of Metabolites

The whole cell broth (WCB) was homogenized by stirring at 400 rpm for 5 to 10 min. Extraction was individually performed for the cell pellet and supernatant of WCB. To extract the compounds (CA, carnosol, ferruginol and pisiferic acid) from the supernatant, 600 µL of homogenized WCB was collected and centrifuged at 11,400× *g* for 5 min. Supernatants were collected and mixed with 22 mL of methanol (99%). The obtained solution was again centrifuged at 4696× *g* for 5 min, and the supernatant was collected to a scintillation vial and stored at −20 °C until the analysis.

The extraction of the cell-associated compounds was performed in a 2 mL bead beating tube. In total, 200 µL of WCB was centrifuged at maximum speed (11,400× *g*) for 5 min, the supernatant was discarded, and residues on the walls of the microtube were cleaned with a cotton swab without touching the pellet. Approximately 1 g of 1 mm glass beads and 600 µL of methanol were added to pellet. Bead beating tubes were placed in a FastPrep-24™ Classic Bead Beating Grinder and Lysis System (MP Biomedicals, Thermo Fisher Scientific, Solon, OH, USA), and a total of four bead beating sessions were performed for 1 min at

6.5 m/s with 5 min interval between each beating cycle. Then, the tubes were vortexed for 30 min in an Eppendorf ThermoMixer® C (Eppendorf AG, Hamburg, Germany) at 2000 rpm and 30 °C, followed by centrifugation at 11,400× *g* for 2 min. After that, 100 µL of supernatant was collected to a scintillation vial and 900 µL of methanol was added to it (10X dilution). The solution was stored at −20 °C until the analysis.

A stock standard solution of 20 mg/L in methanol containing all analytical standards (CA, pisiferic acid, carnosol and the individual solution for ferruginol) was prepared and diluted to concentrations of 5, 2.5, 1.25, 0.625, 0.312 and 0.156 mg/L (except ferruginol, which was diluted at 20, 16, 10, 4, 2 and 1 mg/L). Each standard solution was transferred to a scintillation vial and stored at −20 °C until the analysis. The CA and carnosol used in the analytical analysis were purchased from Sigma Aldrich (Sigma-Aldrich, St. Louis, MO, USA), whereas pisiferic acid and ferruginol were purchased from Santa Cruz Biotechnology (Dallas, TX, USA) and WuXi LabNetwork (Shanghai, China), respectively.

2.4. Quantification of Metabolites

All standards and samples were analyzed by liquid chromatography–electrospray ionization–ultrahigh-resolution–quadrupole time of flight–mass spectrometry (LC-ESI-UHR-QqTOF-MS) with some modifications to the method reported by Oliveira et al., 2015 [12]. A UHPLC from the Bruker Elute series, coupled with a UHR-QqTOF mass spectrometer (Impact II, Bruker Daltonics GmbH, Bremen, Germany) and a BRHSC18022100 Intensity Solo 2 C18 column (100 × 2.1 mm, 2.2 µm, Bruker Daltonik GmbH, Bremen, Germany) at 40 °C, was used for the separation. The injection volume was 5 µL. The mobile phases eluted in the separation column were 0.1% (*v*/*v*) aqueous formic acid (A) and acetonitrile with 0.1% (*v*/*v*) formic acid (B) at a 0.3 mL/min flow rate in gradient mode (30% B at 0 and 2 min; 55% B at 2.5 and 12.7 min; 95% B at 14.1 and 17.45 min; 30% B at 17.7 min; and 0% B at 21.0 min). The MS acquisition was set to negative ionization mode with the following selected parameters: end-plate offset voltage, 500 V; capillary voltage, 3.5 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; and collision cell energy, 5 eV in the mass range from 20 to 1000 *m*/*z*. The MS acquisition was also performed in positive ionization mode with the following selected parameters: end-plate offset voltage, 500 V; capillary voltage, 4.5 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 21.4 to 60 µs; collision cell energy, 10 eV in the mass range from 20 to 1000 *m*/*z*. For the internal mass calibration, sodium formate clusters were used. The standards used for external calibration were CA, carnosol, pisiferic acid and ferruginol.

The detection of each compound mass and the respective reference retention time in an extracted ion chromatogram (EIC) was carried out at 315 ± 0.5 *m*/*z*, RT = 10.4 min for pisiferic acid (negative mode); 329 ± 0.5 *m*/*z*, RT = 6.7 and 7.3 min for carnosol (negative mode); 331 ± 0.5 *m*/*z*, RT = 9.9 min for CA (negative mode); and 287 ± 0.5 *m*/*z*, RT = 15.8 min for ferruginol (positive mode). Since CA converts spontaneously to carnosol, the CA quantities presented in the Results Section were calculated by the sum of the total amounts of CA and carnosol.

3. Results

Shake flask fermentations were conducted before the bioreactor fermentations to ascertain yeast strain growth robustness after genetic engineering. As it can be seen in Figure 2, the engineered yeast strain could consume 97% of the sucrose supplied in 40 h fermentation, achieving good cell growth (wOD 14.88 ± 0.14) (Figure 2). The growth rate

was around 0.2 h^{-1} and the duplication time around 3 h and half (Table 1). Cell viability was kept high from inoculation (>97%) to the end of the fermentation (92.1%; Table 1). However, a significant amount of ethanol accumulated at the end of the fermentation (13.96 ± 0.16 g/L). This was expected due to the Crabtree effect, in which *S. cerevisiae* produces ethanol from glucose in aerobic conditions rather than producing biomass via the tricarboxylic acid (TCA) cycle. Wei et al. (2023) [7] also detected 11.93 g/L of ethanol from c.a. 40 g/L glucose in batch bioreactor fermentation with a CA-producing strain. After sugar depletion, ethanol can itself be used as carbon source to produce the metabolite of interest, in this case, CA [7].

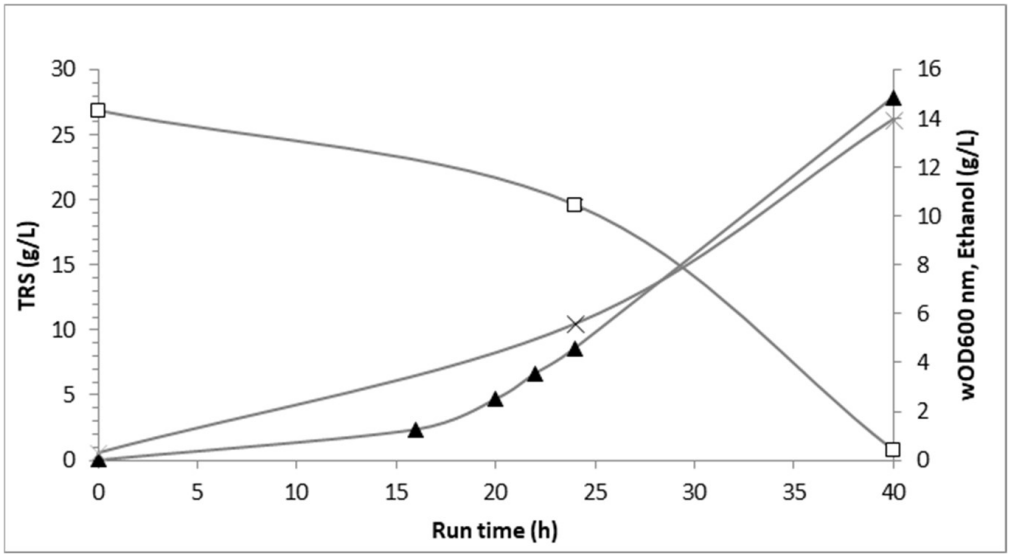


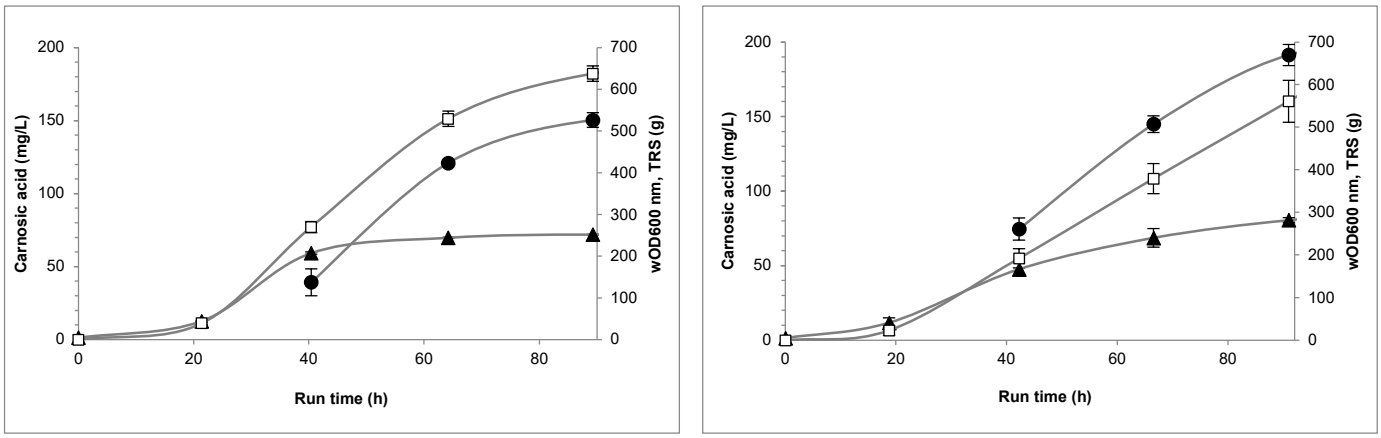
Figure 2. Variation in total recoverable sugars (TRS) (g/L), wOD600 nm and ethanol (g/L) of CA-producing strain during 40 h shake flask fermentations. Legend: –□– TRS (g/L); –▲– washed optical density (wOD600 nm); –x– ethanol (g/L). The data represent the mean of the measurements of 2 independent shake flask fermentations (error bars are negligible).

Table 1. Growth kinetics of CA-producing strain in shake flask fermentations.

	Growth Kinetics
Growth rate (h^{-1})	0.197 ± 0.009
Doubling time (h)	3.52 ± 0.16
TRS consumed (g/L)	26.1
Final cell viability (%)	92.1

The production of CA in the bioreactor was conducted under two different process conditions: (1) an initial feed rate of 12 g/L/h, a maximum feed rate of 26 g/L/h and a DO trigger of 30%; (2) an initial feed rate of 7 g/L/h, a maximum feed rate of 8 g/L/h and a dynamic DO trigger.

Figure 3 shows the variations in wOD600 nm, CA titer and TRS consumption during the fed-batch fermentations. CA production increased from 40 to 90 h, regardless the process conditions. Nevertheless, when an initial feed rate of 7 g/L/h, max. feed rate of 8 g/L/h and dynamic DO trigger were applied, a 27% higher CA titer (191.4 ± 5.0 vs. 150.4 ± 5.0 g/L) and 12% higher wOD600 nm (281.9 ± 6.2 vs. 252.2 ± 0.5) were obtained at day 4 of the fed-batch fermentations (Figure 3). In addition, 13.7% higher TRS were consumed (561 vs. 638 g). As a result, CA yield and productivity were higher at a lower feed rate (Table 2).



(**a**) (**b**)

Figure 3. Variation in CA titer (mg/L), wOD600 nm and cumulative TRS consumed (g) during 90 h fed-batch fermentations at (**a**) DO trigger of 30%, first feed rate of 12 g/L/h and maximum feed rate of 26 g/L/h, and (**b**) dynamic DO trigger, first feed rate of 7 g/L/h and maximum feed rate of 8 g/L/h. Legend: –●– carnosic acid; –▲– washed optical density (wOD600 nm); –□– cumulative total recoverable sugars (TRS). The data represent the mean of the measurements of 2 independent bioreactors, and error bars indicate standard deviations from the mean.

Table 2. CA titer, yield and productivity of CA-producing strain in 4-day fed-batch fermentations (n = 2, independent fermentations).

	Feed Rate 12–26 g/L/h Fixed DO Trigger	**Feed Rate 7–8 g/L/h Dynamic DO Trigger**
Titer (mg/L)	150.4 ± 5.0	191.4 ± 5.0
Yield (mg/g TRS)	0.236	0.341
Productivity (mg/L/h)	1.6	2.0

Figures 4 and 5 illustrate the history plots of fed-batch fermentations in each tested condition. At the beginning of the fermentations, the addition of sugarcane syrup at a slow feed rate (1 g/L/h) allowed the initial ethanol (coming from the inoculum, generated in the second seed bioreactor passage [10]) to be consumed by the culture within 18 h. During this period, the oxygen uptake rate (OUR) increased (up to 55–60 mmol O_2/L/h), DO% decreased and the first DO spike triggered the delivery of the first syrup pulse (10 g/L TRS at different initial feed rates) at 18 h. In the first condition, with a higher initial feed rate of 12 g/L/h, OUR reached more than 160 mmol O_2/L/h and then continuously decreased after the second day, dropping to 30 mmol O_2/L/h by the end of the fermentation (Figure 4). After 3.5 days, DO% increased to 90% and there were no additional DO spikes. On the other hand, in the second process condition, with a dynamic DO trigger, the initial feed rate of 7 g/L/h and max. feed rate of 8 g/L/h, OUR was more stable after the growth phase, ranging between 55 and 90 mmol O_2/L/h (Figure 5), than with serial syrup pulse addition at the maximum feed rate (26 g/L/h) (Figure 4). Moreover, ethanol accumulation during fermentation was higher in the condition with a higher feed rate and a DO trigger of 30% (Figure 4) than in the condition combining a lower feed rate with a dynamic DO trigger (Figure 5). Because of this, fed-batch fermentation was optimal for these process parameters, a dynamic DO trigger, initial feed rate of 7 g/L/h and maximal feed rate of 8 g/L/h, which resulted in higher CA production.

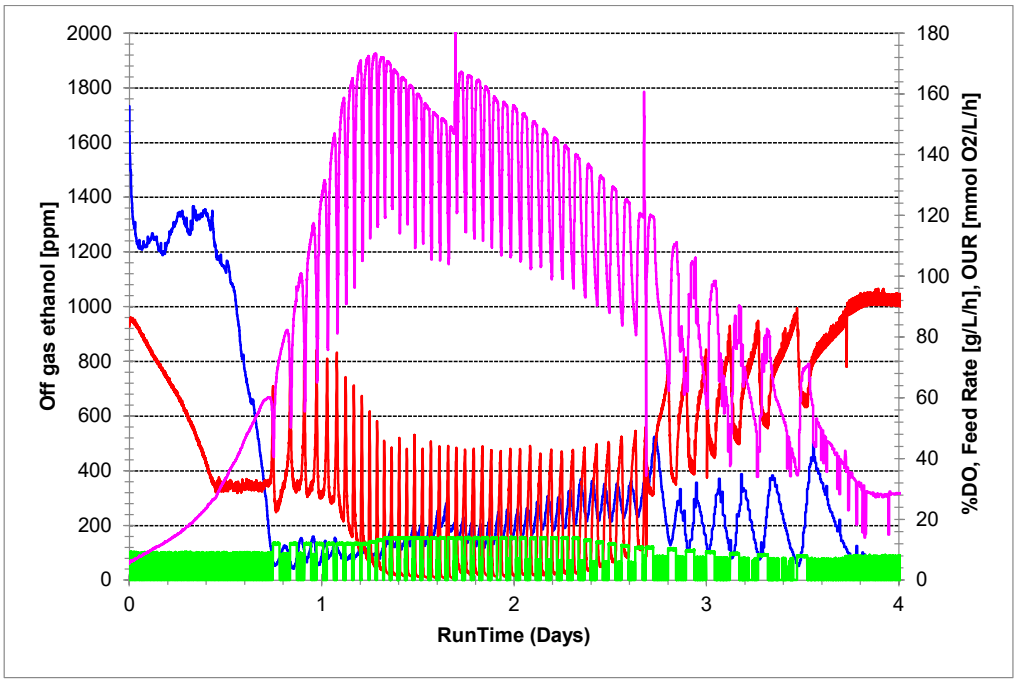


Figure 4. History plots of fed-batch fermentations with feed rate initial of 12 g/L/h, max. feed rate limit of 26 g/L/h and DO% trigger of 30%. Legend: variation in dissolved oxygen (DO%, red line), feed rate (g/L/h, green line), OUR (mmol O_2/L/h, purple line), and off-gas ethanol (ppm, blue line) (1000 ppm off-gas ethanol corresponds to ~5.4 g/L ethanol).

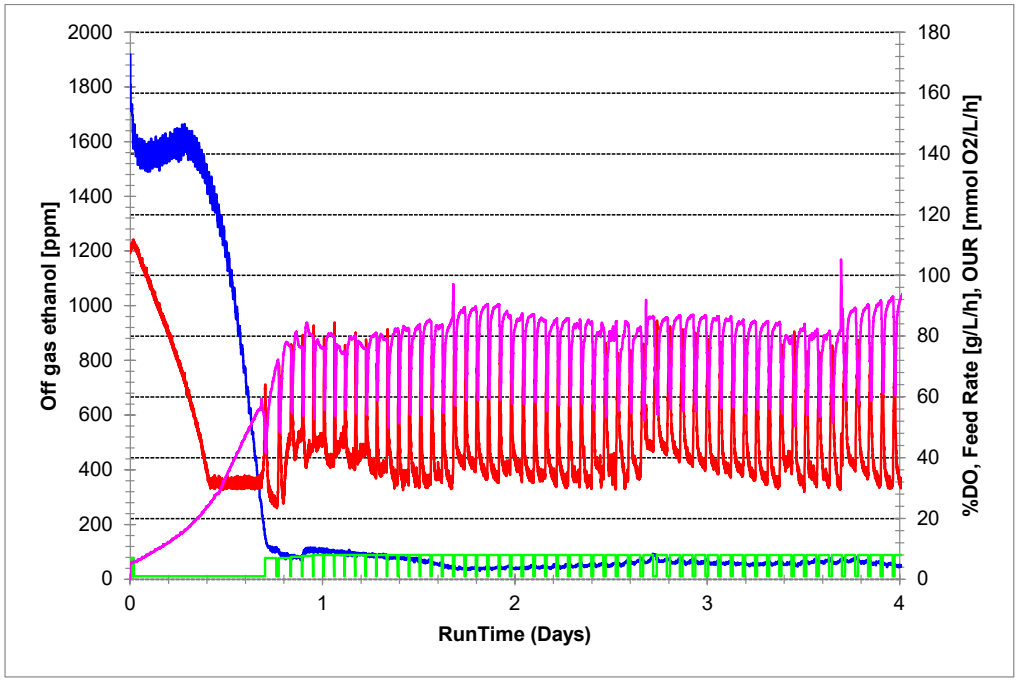


Figure 5. History plots of fed-batch fermentations with feed rate initial of 7 g/L/h, max. feed rate limit of 8 g/L/h and dynamic trigger. Legend: Variation in dissolved oxygen (DO%, red line), feed rate (g/L/h, green line), OUR (mmol O_2/L/h, purple line), and off-gas ethanol (ppm, blue line) (1000 ppm off-gas ethanol corresponds to ~5.4 g/L ethanol).

CA and ferruginol were both detected inside the cells and in the supernatant, but they were mainly intracellular molecules. More than 92% of CA was determined inside the cells in the best fed-batch fermentation condition (a dynamic DO trigger, the first feed rate of 7 g/L/h and the maximum feed rate of 8 g/L/h) (Figure 6). This corresponded to

179 mg/L of intracellular CA, the concentration of which might limit the overproduction of CA due to the expected toxicity of CA on *S. cerevisiae* cells. In the European Union, rosemary extracts that contain approximately 50% of total CA and carnosol (g/100 g extract) are added to food and beverages at levels of up to 400 mg/kg as antioxidant and antimicrobial supplements [13,14]. A previous report also showed that the minimal inhibitory concentration of rosemary oil against *Escherichia coli* was above 6.4 mg/L [14]. Moreover, the addition of 8 to 10 mg/L CA in the culture improved the antimicrobial actions on bacteria such as *Staphylococcus aureus*, *Enterococcus faecium* and *Enterococcus faecalis* [2]. The toxicity of CA towards the yeast *Candida albicans*, especially at levels above 50 mg/L, has been reported [15].

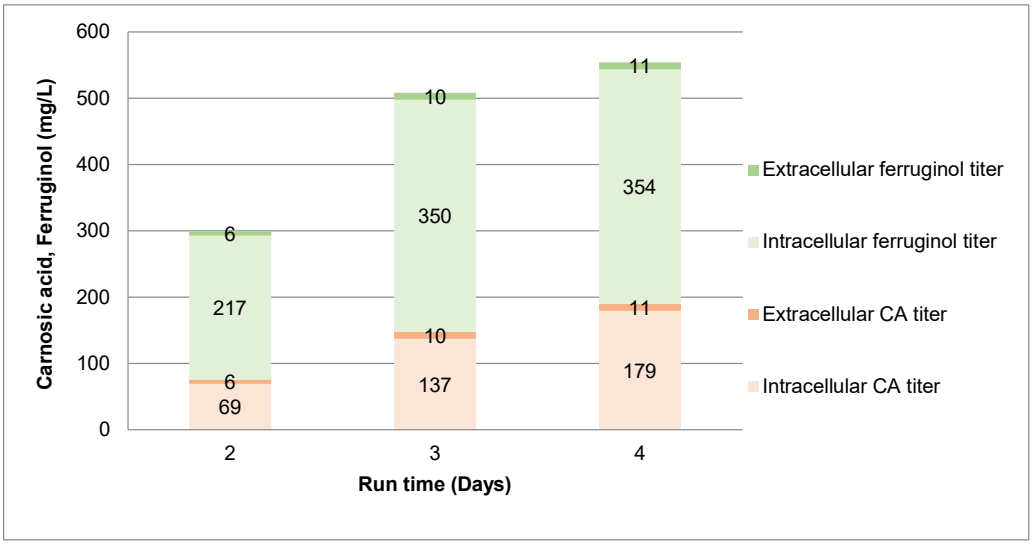


Figure 6. Percentage of intracellular and extracellular CA and ferruginol titers (mg/L) at days 2, 3 and 4 of optimized fed-batch fermentation (dynamic DO trigger, first feed rate of 7 g/L/h and maximum feed rate of 8 g/L/h).

Ferruginol was 364.4 mg/L at day 4 of the optimized fed-batch fermentation (Figure 6). The amount of ferruginol in the culture broth, mostly in intracellular form (91.7% of total ferruginol), was 1.9-fold higher than the CA quantity, which indicates that the low conversion rate of the precursor ferruginol is a limiting step for the improvement in CA productivity [7]. Another metabolite generated in the CA production pathway is pisiferic acid, which was not detected in the fermentation broth. Pisiferic acid is a secondary product in CA biosynthesis, also being derived from the precursor ferruginol (Figure 1). The absence of this metabolite in the fermentation broth indicates full ferruginol availability for CA biosynthesis.

4. Discussion

CA has attracted increasing attention in recent years due to its remarkable biological and biomedical properties [1,16,17]. The levels of CA in field-grown rosemary plants present seasonal changes, with a tendency for losses in response to environmental stress conditions, such as high temperatures or precipitation [1]. Despite its importance, this compound remains largely inaccessible by chemical synthesis. Thus, the development of biotechnological methods for alternative CA production is of high interest, which may offer a more controlled and sustainable source. The complete biosynthetic pathway of CA has been elucidated in recent years and reconstituted in *S. cerevisiae* [3,5,6]. Up to 24.65 mg/L of CA was obtained from the engineered *S. cerevisiae* yeast strains in small-scale experiments [7]. The obtained amounts are still beyond the targets needed to develop

yeast-sourced CA into a commercial product. However, apart from a single work being conducted on it [7], the fermentation process remains underdeveloped.

Thus, this work aimed to study the feasibility of producing CA by yeast fermentation from a sustainable feedstock, sugarcane syrup. Sugarcane syrup contains high concentration of fermentable sugars, mainly sucrose, and lower amounts of glucose and fructose, is low cost, and is produced from a renewable source, the sugarcane, largely available in tropical regions [10]. It has also a range of minerals (mg/L), such as calcium, magnesium, and potassium, which can be used by the yeast during fermentation [11]. Sugarcane syrup is an important carbon feedstock in the industrial production of many value-added molecules, obtained by yeast fermentation, such as the terpenoids β-farnesene and artemisinic acid (antimalarial drug precursor). Namely, g/L titers are reported for farnesene (130 g/L) and artemisinic acid (25 g/L) produced from sugarcane feedstock in engineered yeast *S. cerevisiae* through optimized fed-batch fermentation [18–20]. *S. cerevisiae*, engineered with the CA pathway, was used to produce CA in a similar fed-batch fermentation process as used in our previous work, in which β-farnesene was stably produced at the g/L scale from sugar cane syrup for 13 days [10]. The first step was to confirm the suitable growth and sugar consumption capacity of the CA engineered strain in small-scale experiments (Figure 2). Then, the impact of higher (12–26 g/L/h) and lower (7–8 g/L/h) sugarcane syrup feed rates on CA production, delivered by DO spikes (using a fixed or dynamic trigger, respectively), was studied. Lower sugar feed rates resulted in better fermentation performance and in a 1.3-fold improvement in CA productivity (2.0 mg/L/h vs. 1.6 mg/L/h) and 1.4-fold improvement in CA yield from sugarcane sugars (0.341 mg/g vs. 0.236 mg/g) (Table 2). High cell density was achieved under both feed rate conditions, with optical densities above 250 at the end of the fermentation (Figure 3). CA concentration reached 150.4 and 191.4 mg/L in the different bioreactor conditions tested (Table 2). The CA production levels reported in the literature are mainly for shake flask experiments. In *S. cerevisiae*, the CA levels ranged from 1.00 mg/L to a maximum of 24.65 mg/L [3,5–7]. When performing 5 L fed-batch fermentation, the CA titer could be improved by 3-fold, i.e., up to 75.18 mg/L [7]. More recently, by using a different yeast host, *Candida tropicalis*, a CA titer of 78.24 mg/L was obtained [21]. In the work presented herein, CA concentration reached 191.4 mg/L in the best bioreactor condition in 4-day fermentation (Figure 3), which corresponds to a 2.5-fold increase compared to the higher CA titers reported in the literature [7,21]. Moreover, the previous bioreactor titer (75.18 mg/mL) was obtained in a longer fed-batch fermentation process (7.5 days) using a two-stage feeding strategy, consisting of glucose and ethanol, both used as carbon sources [7], while in this work, a higher CA titer (191.4 mg/mL) was obtained in a shorter bioreactor process, using a more sustainable carbon source, sugarcane syrup. Besides advances in the CA fermentation process, this work also identified important challenges in the production of CA. CA was not secreted, which may impose toxicity on the yeast cells and restrict yeast production due to the pronounced CA antimicrobial activity [13,14]. In addition, ferruginol conversion was found to be a limiting step in the CA biosynthetic pathway. At the end of the fermentation, the ferruginol titer was almost double the CA concentration (Figure 6). In a previous study, ferruginol was also higher (3.7-fold) than CA concentration in fed-batch fermentation [7]. In that study, *Salvia pomifera* hydroxy ferruginol synthase CYP76AH24 was employed. Although the hydroxy ferruginol synthase used in this work from *R. officinalis* seems more efficient than the one from *S. pomifera*, in subsequent studies, these enzymes must be replaced by other enzymes with higher catalytic efficiency, or they should be engineered with that aim. Moreover, a suitable transport mechanism for the secretion of CA from the intracellular to the extracellular space will be necessary to improve CA production, which will also facilitate the CA downstream process. In addition, the reduction in CA toxicity in the culture broth may be achieved

by using extractive fermentation methods. Two-phase fermentation technology has been widely applied in terpene fermentation due to its great advantages, including enhanced productivity, a reduction in toxicity to microbial cells, the alleviation of feedback inhibition, and the prevention of product degradation and loss [22]. A diverse range of solvents, including n-dodecane, isopropyl myristate, methyl oleate, n-decane, oleyl alcohol, and n-hexane, along with adsorbents such as resins, have been employed to enhance the extraction efficiency of terpenes from the culture medium. These methods have been successfully applied to the recovery of monoterpenes (e.g., geraniol, linalool, limonene, perillyl alcohol), sesquiterpenes (e.g., farnesene, amorphadiene, patchoulol, bisabolene, nerolidol), diterpenes (e.g., taxadiene, miltiradiene, sclareol, geranylgeraniol, retinol), triterpenes (e.g., squalene, betulinic acid, damarenediol-II, protopanaxadiol), and tetraterpenes (e.g., lycopene). The application of this technology has been reported to significantly enhance terpene titers, with increases of 167-fold for geraniol, 41-fold for limonene, 8.5-fold for amorphadiene, 3.38-fold for β-caryophyllene, 10-fold for miltiradiene, and 40-fold for taxadiene [22].

In summary, sugarcane syrup was shown to be a suitable feedstock to produce CA from *S. cerevisiae* in 2 L fed-batch fermentation. By reducing syrup feed rate and applying a dynamic DO trigger, the CA titer was increased by 27%. The optimized process led to the production 191.4 mg/L CA, mainly intracellularly, which is the highest CA titer reported in yeast to date. However, further improvements in the CA synthetic pathway will be necessary before reaching higher titers by fermentation process optimization.

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