CATÓLICA
ESCOLA SUPERIOR DE BIOTECNOLOGIA
PORTO

EFFECT OF POLYPHENOLS ON *SACCHAROMYCES CEREVISIAE* FED-BATCH FERMENTATION

Thesis submitted to *Universidade Católica Portuguesa* to attain the degree of PhD in Biotechnology, with specialization in Microbiology

Luís Carlos Rocha Carvalho

August 2023

CATÓLICA
ESCOLA SUPERIOR DE BIOTECNOLOGIA
PORTO

EFFECT OF POLYPHENOLS ON *SACCHAROMYCES CEREVISIAE* FED-BATCH FERMENTATION

Thesis submitted to *Universidade Católica Portuguesa* to attain the degree of PhD in Biotechnology, with specialization in Microbiology

Luís Carlos Rocha Carvalho

Supervisor: ***Carla Cristina Marques Oliveira, PhD*** (Universidade Católica Portuguesa)

Co-supervisors: ***Erdem Carsanba, PhD*** (Amyris BioProducts Portugal)

and ***Ana Lúcia Silva Oliveira, PhD*** (Universidade Católica Portuguesa)

August 2023

Eu dedico esta tese a quem
esteve ao meu lado neste percurso.

This work was co-financed by European Regional Development Fund (ERDF) through the Operational Program for Competitiveness and Internationalization (POCI) supported by the Amyris Bio Products Portugal, Unipessoal Lda and Escola Superior de Biotecnologia – Universidade Católica Portuguesa through Alchemy project 'Capturing High Value from Industrial Fermentation Bio Products (POCI-01-0247-FEDER-027578)'. Thanks are also due to Universidade Católica Portuguesa and FCT/MCT for the financial support for the CBQF (project UIDB/50016/2020) research unit, through national funds and where applicable co-financed by the FEDER, within the PT2020 Partnership Agreement.

Resumo

A produção industrial de β-farneseno é alcançada através da fermentação semi-contínua de *Saccharomyces cerevisiae*, principalmente utilizando xarope de cana-de-açúcar como matéria-prima. No entanto, a presença de fenóis no xarope de cana-de-açúcar pode impactar o desempenho da levedura. Esta tese teve como objetivo estudar a evolução dos fenóis ao longo da produção de β-farneseno por *S. cerevisiae* em fermentações de biorreator utilizando xarope de cana-de-açúcar, avaliar o impacto dos fenóis neste processo, e explorar o potencial de xarope de beterraba, uma matéria-prima alternativa com menos fenóis, na produção de β-farneseno através de um processo similar.

Os fenóis presentes no xarope e no caldo fermentativo foram identificados e quantificados. O xarope de cana-de-açúcar apresentou 50.7 mg/L de conteúdo fenólico total (CFT), compreendendo ácidos hidroxibenzóicos, ácidos hidroxicinámicos e flavonoides. Entre os dois modelos cinéticos avaliados, o modelo Weibull exibiu um ajuste superior ($R^2 \geq 0.85$) e descreveu com precisão a acumulação fenólica durante a fermentação semi-contínua de 13 dias em biorreatores de 2 L. A concentração da maioria dos fenóis aumentou até ao dia 3, estabilizando até ao fim da fermentação. No entanto, hidroxibenzaldeído e os ácidos protocatecuico, cafeico, ferúlico e *p*-cumárico diminuíram após o segundo dia, indicando a sua metabolização pela levedura. Este trabalho fornece um modelo que pode ser aplicado para descrever a acumulação fenólica em processos similares.

Para investigar a influência dos fenóis na fermentação, os fenóis do xarope de cana-de-açúcar foram removidos com carvão ativado. O processo de purificação de xarope foi otimizado com um desenho composto central, alterando o tempo de contacto e o tipo e a concentração de carvão. A otimização da purificação convergiu na condição com pastilhas de carvão a 115 g/L e com 12.5 h de tempo de contacto, e removeu 96.7 % dos fenóis e recuperou 43.7 % da massa de xarope. A introdução de xarope purificado em fermentações apresentou resultados divergentes em função da escala. Apesar de em frascos ter havido uma melhoria de 11 % na produtividade de β-farneseno, em biorreatores não foi detetado nenhum aumento. Em contraste, um aumento de 12 % na produtividade de biomassa foi exclusivo dos biorreatores. Portanto, para as condições testadas, os fenóis não influenciaram a produção de β-farneseno em grande escala.

Para além disso, a produção de β-farneseno utilizando uma matéria-prima alternativa, xarope de beterraba, foi bem-sucedida. Apesar de apresentar CFT menor (21.3 mg/L) comparado com o xarope de cana-de-açúcar (50.7 mg/L), o xarope de beterraba apresentou elevada concentração de glucose (193 g/L) e hidroximetilfurfural (HMF; 1.4 g/L). A preparação do inóculo no segundo passo em reator necessitou de otimização devido ao crescimento lento da levedura e a densidade celular desejada foi atingida em fermentação semi-contínua com 40 g/L de açúcares de xarope de beterraba e com pulsos de 10 g/L. Após 8 dias, a fermentação semi-contínua resultou em 127 g/L de concentração de β-farneseno, com 19.44 % de rendimento acumulado e 1.70 g/L/h de produtividade acumulada, demonstrando a viabilidade de usar xarope de beterraba nesta fermentação.

Em conclusão, esta tese destacou a relação entre os fenóis e o processo de fermentação semi-contínuo de β-farneseno destacando o aumento fenólico no caldo fermentativo. Notavelmente, fenóis específicos foram metabolizados pela levedura. Os efeitos dos fenóis do xarope de cana-de-açúcar em *S. cerevisiae* foram dependentes da escala de fermentação. Mesmo sendo valiosos para a mitigação do stress oxidativo na levedura, a remoção dos fenóis não melhorou a produtividade da fermentação em biorreator. Por outro lado, a utilização do xarope de beterraba como matéria-prima pode permitir a produção de β-farneseno em climas amenos sem cultivo extensivo de cana-de-açúcar, fomentando a produção sustentável em regiões diversificadas.

Palavras-chave: *Saccharomyces cerevisiae*, fenóis, β-farneseno; fermentação em biorreator, atividades antioxidante e antimicrobiana, xarope de cana-de-açúcar, xarope de beterraba.

Abstract

The industrial production of β-farnesene is traditionally achieved through *Saccharomyces cerevisiae* fed-batch fermentation, often employing sugarcane syrup as the feedstock. However, the presence of phenolic compounds in sugarcane syrup potentially impacts yeast performance. This thesis aimed to study the evolution of phenolic compounds over β-farnesene production by *S. cerevisiae* in bioreactor fermentations with sugarcane syrup, assess the phenolic compounds' impact on this process, and explore the potential of beet syrup, a lower phenolic content alternative feedstock, for β-farnesene production via a similar fermentation process.

The phenolic compounds present within both the sugarcane syrup and the fermentation broth were systematically identified and quantified. Sugarcane syrup presented 50.7 mg/L of total phenolic content (TPC), comprising hydroxybenzoic acids, hydroxycinnamic acids and flavonoids. Among two kinetic models assessed, the Weibull model exhibited superior fitting capacity ($R^2 \geq 0.85$) and accurately depicted phenolic compounds' accumulation during the 13-day fed-batch fermentation in 2 L bioreactors. The concentration of most phenolic compounds ascended until day three, stabilizing until the end of the fermentation process. Nevertheless, hydroxybenzaldehyde and protocatechuic, caffeic, ferulic and *p*-coumaric acids declined after the second day, indicating yeast metabolism. This work provides a model that can be applied to describe the phenolic accumulation during similar fed-batch processes.

To investigate the phenolic influence on the fermentation, sugarcane syrup phenolic compounds were effectively removed with activated charcoal. The syrup purification process was optimized with a central composite design, varying the type of charcoal, concentration of charcoal and contact time. Optimization yielded purification conditions with charcoal pellets at 115 g/L and 12.5 h of contact time, while removing 96.7 % of phenolic compounds and recovering 43.7% of syrup mass. Purified syrup introduction into fermentations displayed divergent scale-dependent outcomes. Although in shake-flasks there was an 11 % enhancement in β-farnesene productivity, no significant increase was detected in bioreactors. In contrast, an increment in biomass productivity was exclusive to bioreactors, registering an increase of 12 %. Therefore, for the conditions tested, the phenolic compounds did not influence the β-farnesene production at large-scale.

Moreover, β-farnesene production using an alternative feedstock, sugarbeet syrup, was successful. Despite presenting lower TPC (21.3 mg/L) compared to sugarcane syrup (50.7 mg/L), beet syrup contained high glucose (193 g/L) and hydroxymethylfurfural (HMF; 1.4 g/L) concentrations. Inoculum production in the second bioreactor step required optimization due to slow growth and the desired cell density was achieved with a fed-batch mode with initial 40 g/L of sugars from beet syrup and 10 g/L of sugar pulses. Over 8 days, fed-batch fermentation yielded 127 g/L β-farnesene concentration, with 19.44 % cumulative yield and 1.70 g/L/h cumulative productivity, affirming the viability of using beet syrup as the fermentation feedstock.

In conclusion, this thesis showcased the dynamic relationship between phenolic content and β-farnesene fed-batch process highlighting an increase in the fermentation broth's phenolic content. Notably, specific phenolic compounds were metabolized by the yeast. The effects of sugarcane syrup phenolic compounds on *S. cerevisiae* were dependent on the fermentation scale. While valuable for mitigating yeast oxidative stress, phenolic compounds' removal did not enhance bioreactor fermentation productivity. On the other hand, the utilization of beet syrup as feedstock may enable β-farnesene production in mild climates without extensive sugarcane cultivation, fostering sustainable production across diverse regions.

Keywords: *Saccharomyces cerevisiae*, phenolic compounds, β-farnesene; bioreactor fermentation, antioxidant and antimicrobial activities, sugarcane syrup; sugarbeet syrup.

Acknowledgments

Quero agradecer a todos os que contribuíram, de forma direta ou indireta, para a realização desta tese.

Em primeiro lugar, quero agradecer às instituições que me acolheram pela oportunidade de realizar este percurso.

Nomeadamente, à Escola Superior de Biotecnologia, Centro de Biotecnologia e Química Fina e à Amyris BioProducts Portugal por me fornecerem inesgotáveis recursos, incluindo as condições do meu local de trabalho e o financiamento do projeto Alchemy.

Ainda, à Católica Business School pela oportunidade de aprender a resolver problemas de uma forma alternativa, mas sistemática.

De seguida, agradeço às pessoas que me acompanharam neste percurso profissional.

Aos meus orientadores, Carla Oliveira, Ana Oliveira e Erdem Carsanba por tudo o que me ensinaram ao longo destes anos, por me encaminharem para uma direção adequada, pelos debates de ideias e pela motivação.

À Professora Manuela, pela oportunidade de realizar este projeto e pelas mensagens de ânimo ao longo deste percurso.

À minha equipa de Fermentações, por toda a aprendizagem e suporte que me deram e porque, devido à nossa louvável e enorme entreajuda, esta tese é também em parte vossa.

À Anita, Poliana e Soraia, pela incrível ajuda independentemente da altura.

E ainda, quero agradecer às pessoas que me apoiaram fora do local de trabalho, mas que também contribuíram para que esta tese fosse possível.

Aos meus pais, por serem os responsáveis principais por esta jornada me trazer a este momento e por todo o suporte, amor e ajuda durante todo este tempo. Sem vocês nada disto seria possível.

À minha Marta, pelo carinho, suporte, compreensão e incentivo incansáveis ao longo destes anos e por continuares a fazer de mim uma pessoa melhor.

Ao meu irmão André e à minha cunhada Flávia, pelo ânimo e motivação que me deram.

Às meninas do “Passear”, pela companhia em todos os momentos, pelos milhares de músicas desfrutadas a lavar reatores, e pelo companheirismo e amizade que me aqueceu o coração.

Aos homens do “Cadeira”, pela descontração, parvoíce e camaradagem que me fizeram sempre sentir que posso contar convosco.

Aos “Leves” e “Sardinhas”, pelas conversas que me suavizaram o percurso, onde quer que estivessem.

Aos “Coxos”, pelos momentos coletivos e individuais que me fazem querer pertencer a esta equipa de jogadores extremamente talentosos.

À Guida e ao Bruno, por me ajudarem a descontrair e a aperceber a importância da amizade.

Table of contents

List of figures

List of tables

Abbreviations

	$[M-H]^-$	Accurate mass
	μ	Specific growth rate
A	ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
	ANOVA	One-way analysis of variance
	ATP	Adenosine triphosphate
C	CCD	Central composite design
D	d	Days
	DNA	Deoxyribonucleic acid
	DO	Dissolved oxygen
	DPPH	2,2-Diphenyl-1-picrylhydrazyl
E	EGCG	Epigallocatechin gallate
F	FID	Flame ionization detector
G	GAE	Gallic acid equivalents
	GC	Gas chromatography
H	h	Hours
	HBA	Hydroxybenzoic acids
	HCA	Hydroxycinnamic acids
	HMF	Hydroxymethylfurfural
	HPLC	High-performance liquid chromatography
I	IC HPAE-PAD	Ion Chromatography High-Performance Anion-Exchange Chromatography coupled with Pulse Amperometric Detection
	IC_{50}	Half maximal inhibitory concentration
L	LC-ESI-UHR-QqTOF-MS	Liquid Chromatography – Electrospray Ionization – Ultrahigh-Resolution - Quadrupole Time of Flight – Mass Spectrometry

	LC-MS	Liquid chromatography mass spectrometry
	LSD	Least significant different
M	MIC	Minimum inhibitory concentration
	min	Minutes
N	NA	Not available
	NADH	Nicotinamide adenine dinucleotide hydrogen
	ND	Not described
	$NO^{\cdot}$	Nitric oxide radical
	$NO_2^{\cdot}$	Nitrogen dioxide radical
O	$O_2^{\cdot -}$	Superoxide radical
	$OH^{\cdot}$	Hydroxyl radical
	ORAC	Oxygen radical absorbance capacity
P	PAD	Phenylacrylic acid decarboxylase
	PE	Phycoerythrin
	Per-CP	Peridinin chlorophyll protein complex
	P_P	Product productivity
	PSA	Post-sterile addition
	P_X	Biomass productivity
	R^2	Coefficient of determination
R	RFU	Relative fluorescent unit
	RID	Refractive Index Detector
	RMSE	Root mean square error
	$RO^{\cdot}$	Alkoxyl radical
	$RO_2^{\cdot}$	Peroxyl radical
	ROS	Reactive oxygen species

T	TPC	Total phenolic content
	TRS	Total reducing sugars
W	WCB	Whole cell broth
	wOD	Washed optical density
Y	$Y_{P/S}$	Yield of product to substrate
	$Y_{X/S}$	Yield of biomass to substrate

Chapter 1

Introduction

Background

The present thesis was developed within the scope of the Alchemy Project, a collaboration between the Centre of Biotechnology and Fine Chemistry (Universidade Católica Portuguesa) and the biotechnology company Amyris Inc. The work of Amyris is specialized in the fermentation of the engineered yeast *Saccharomyces cerevisiae* to produce value-added molecules, with β-farnesene being their primary product. This is achieved through industrial fed-batch fermentation, where sugarcane syrup serves as the feedstock. This syrup contains phenolic compounds in its composition, which have proven antioxidant and antimicrobial activities. Thus, it was hypothesized that the phenolic compounds in sugarcane syrup may have influence on the yeast metabolism and, consequently, on the fermentation performance. Therefore, the study of the impacts of phenolic compounds on the β-farnesene fermentation may contribute to exploit new paths for improving this established fermentation process.

Scope and outline

This thesis aimed to study the evolution of phenolic compounds over β-farnesene production by *S. cerevisiae* in bioreactor fermentations supplied with sugarcane syrup, the impact of phenolic compounds on this process, and explore the potential of a lower phenolic content alternative feedstock, beet syrup, for β-farnesene production through a similar fermentation method. To study the effect of these compounds on the fermentation and to scale-up the solutions proposed, both shake-flask and bioreactor fermentations were performed. The specific objectives of this work were: the characterization of phenolic compounds in sugarcane syrup and in the fermentation broth of the β-farnesene fed-batch fermentation process; the identification of the impact of these phenolic compounds on the yeast fermentation through the removal of the phenolic compounds from sugarcane syrup and its supply to the fermentation process; and evaluation of the feasibility in the utilization of a feedstock with decreased phenolic composition, beet syrup, in the β-farnesene production process.

The thesis outline is depicted in Figure 1. Briefly, the introductory section (**Chapter 1**) provides a literature review on the current knowledge regarding the effects of phenolic compounds on *S. cerevisiae*, with the point of view of the fermentation process and, additionally, the strategies for the mitigation of the growth and production inhibition by phenolic compounds. To attain the objectives described, the experimental side of this thesis was divided into three different parts: i) phenolic compounds modulation in β-farnesene fed-batch fermentation using sugarcane syrup as feedstock (**Chapter 2**); ii) removal of phenolic compounds from sugarcane syrup and impact on *S. cerevisiae* fermentation (**Chapter 3**); and iii) production of β-farnesene from sugarbeet syrup by *S. cerevisiae* in fed-batch fermentation (**Chapter 4**). Finally, the overall conclusions and future perspectives are presented in the section of the final remarks (**Chapter 5**).

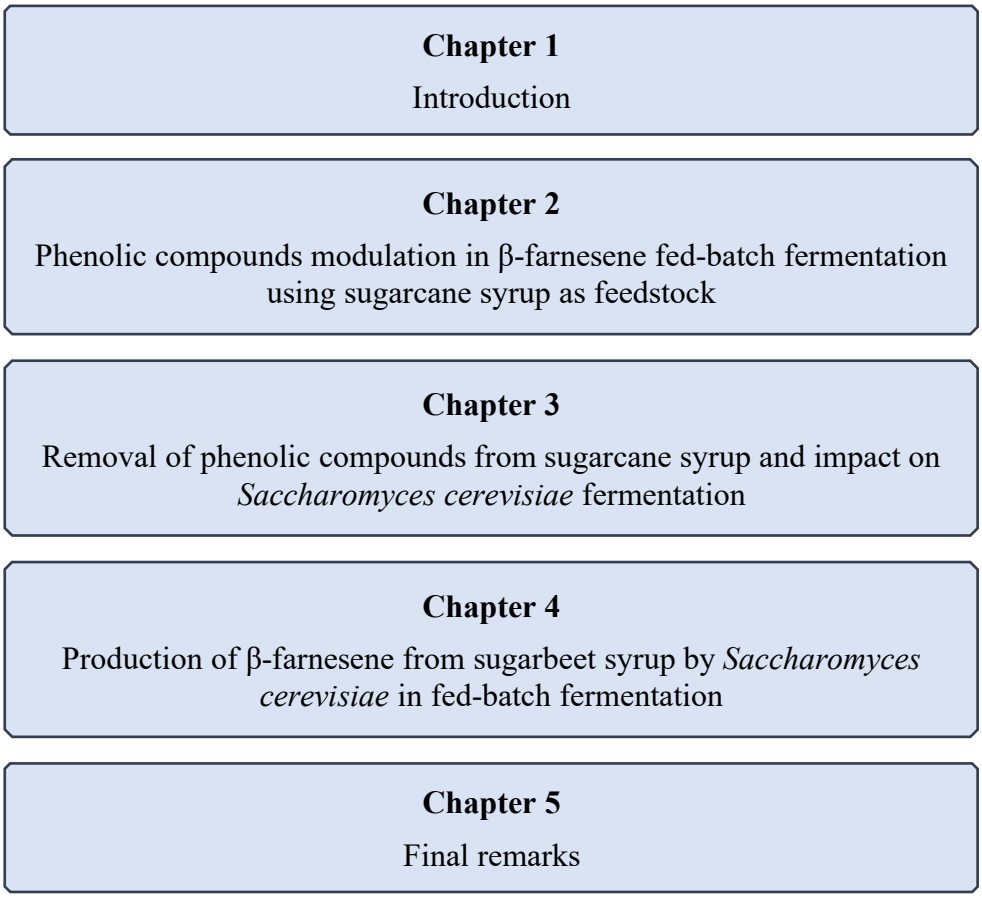

Figure 1. Outline of the thesis.

To start this work, the phenolic compounds of the sugarcane syrup were identified and quantified (**Chapter 2**). Then, the same quantification was performed for the fermentation broth during the 13 days of the Amyris fed-batch fermentation process simulated in the laboratory in 2 L bioreactors. The phenolic evolution was modulated according to two mathematical calculations: a mass balance and the Weibull model.

Afterward, the aim was to assess the effects of the sugarcane syrup phenolic compounds on the yeast. For this, the phenolic compounds were removed from the syrup using activated charcoal and the purification process was optimized using a central composite design (**Chapter 3**). Then, the purified syrup was added to β-farnesene fermentations alongside non-purified syrup. This allowed to identify the effects of the removal of phenolic compounds on the yeast and the fermentation parameters.

Lastly, to diversify the feedstock options and lower the carbon emissions for the β-farnesene fermentation process, the use of a different feedstock in the β-farnesene production

was evaluated (**Chapter 4**). Sugarbeet syrup was selected due to the availability of sugarbeet in milder climates, such as in Europe. This feedstock was expected to have a different phenolic profile than sugarcane syrup, and the viability of using beet syrup as an alternative feedstock was assessed.

Scientific Output

Chapter 2

Poster in national conference – Microbiotec 2021

Luís Carlos Carvalho, Ana L.S. Oliveira, Erdem Carsanba, Manuela Pintado & Carla Oliveira. 23rd to 26th of November 2021. Model for polyphenols prediction in yeast fed-batch fermentation using sugarcane syrup as feedstock. *Microbiotec 2021*. Lisbon, Portugal.

Oral Presentation and Poster in international conference – BioIberoAmerica 2022

Luís Carlos Carvalho, Ana L.S. Oliveira, Erdem Carsanba, Manuela Pintado & Carla Oliveira. 7th to 9th of April 2022. Modulation of polyphenols in β-farnesene fed-batch fermentation. *BioIberoAmerica 2022*. Braga, Portugal.

Research Article in peer-reviewed journal – Industrial Crops and Products

Luís Carlos Carvalho, Ana L.S. Oliveira, Erdem Carsanba, Manuela Pintado & Carla Oliveira. 2022. Phenolic compounds modulation in β-farnesene fed-batch fermentation using sugarcane syrup as feedstock. *Industrial Crops and Products*. 188. 115721. https://doi.org/10.1016/J.INDCROP.2022.115721.

Chapter 3

Poster in national conference – Encontro Ciência 2021

Luís Carlos Carvalho, Ana L.S. Oliveira, Erdem Carsanba, Manuela Pintado & Carla Oliveira. 28th to 30th June 2021. Detoxification of phenols in sugarcane syrup for improved yeast fermentation. *Encontro Ciência 2021*. Lisbon, Portugal.

Oral Presentation in international conference – ICP 2023

Luís Carlos Carvalho, Ana L.S. Oliveira, Erdem Carsanba, Manuela Pintado & Carla Oliveira. 3rd to 6th of July 2023. Optimizing the removal of phenolic compounds from sugarcane syrup with activated charcoal. *International Conference on Polyphenols 2023*. Nantes, France.

Literature review

1. Definition and classification of phenolic compounds

Phenolic compounds are a diverse group of thousands of natural molecules resulting from the secondary metabolism of plants (Albuquerque et al., 2021). These molecules are essential to the plants because they provide structural support and protect them against solar radiation, pathogens and predators, such as parasites and insects (Albuquerque et al., 2021; de la Rosa et al., 2019; Vuolo et al., 2019). Moreover, the color and aroma of fruits and vegetables originate from the abundance of phenolic compounds (Vuolo et al., 2019). They are ubiquitous molecules and can be found in high quantities in the seeds, skins of fruits or leaves of vegetables and either exist in a soluble or a bound form (Gan et al., 2019; Kumar and Goel, 2019).

The structure of phenolic compounds is characterized by having one or more hydroxyl groups attached directly to an aromatic ring and the molecules can be simple or polymerized (Vuolo et al., 2019). Based on their chemical structure, phenolic compounds are divided into classes (Figure 2), which include phenolic acids, flavonoids, xanthones, stilbenes, tannins, lignins, and quinnones (Gan et al., 2019; Vuolo et al., 2019). In addition, the classification of phenolic compounds is divided into monophenols, when one hydroxyl group is present, or polyphenols, when multiple hydroxyl groups are connected to the aromatic ring.

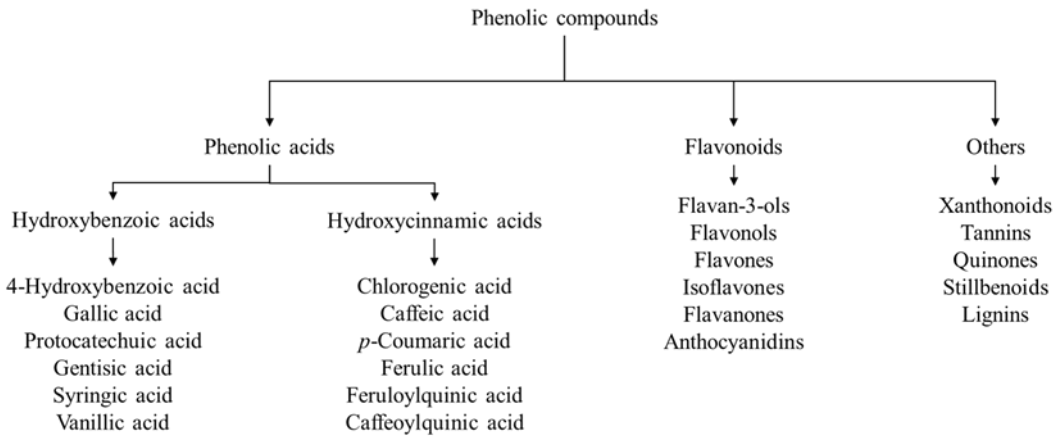

Figure 2. Classification of phenolic compounds based on the phenolic rings and their connecting elements and examples of the main phenolic acids commonly encountered (adapted from Gan et al., 2019).

Phenolic acids represent a principal phenolic class, comprising compounds characterized by the presence of a carboxylic group connected to an aromatic ring (Kumar and Goel, 2019). Typically, they are found with additional amide, ester, or glycoside groups attached. Most phenolic acids can be categorized into two primary subclasses: hydroxybenzoic and hydroxycinnamic acids (Vuolo et al., 2019). Hydroxybenzoic acids (HBA) have the basic structure of C6-C1. Some examples of HBA are 4-hydroxybenzoic acid, protocatechuic acid, gentisic acid, vanillic acid, syringic acid and gallic acid (Vuolo et al., 2019). On the other hand, hydroxycinnamic acids (HCA) have a C6-C3 carbon backbone, with a double bond in the chain. Examples of HCA encompass *p*-coumaric acid, ferulic acid and caffeic acid (Kumar and Goel, 2019).

Flavonoids, the most abundant and diverse class of phenolic compounds, are characterized by a fundamental chemical structure known as C6-C3-C6, comprising a three-carbon connection that links two aromatic rings. This unique carbon connection gives rise to a third benzoic ring, which becomes attached to one of the two primary rings. Subclasses of flavonoids include flavonols, flavones, flavanones, flavanols, isoflavones, and anthocyanidins (Dias et al., 2020; Vuolo et al., 2019). Flavonoids are biosynthesized in plants through the phenylpropanoid or the polyketide pathways. The composition of flavonoids in each plant depends on multiple factors, such as environmental conditions, physical injuries and which hormones are present (Dwivedi et al., 2020). Additionally, most flavonoids occur linked to sugar glycosides. Flavonoid glycosides have improved cell membrane solubility and present increased plant protection (Šamec et al., 2021). Flavonoids universally possess antioxidant properties, and a considerable portion of them present protective effects against human diseases, including anti-inflammatory, neuroprotective, antiviral, and anticancer properties (Albuquerque et al., 2021). Moreover, specific flavonoids such as catechin, naringin, and anthocyanins have been found to exhibit notable antimicrobial activity (Albuquerque et al., 2021).

Other groups of phenolic compounds include xanthonoids, stilbenoids, tannins, lignins, and quinnones. Xanthonoids have the basic structure of C6-C1-C6, with the rings directly connected with each other. They can have antimicrobial, antioxidant, and antiviral activities (Maphetu et al., 2022). Stilbenoids are represented with a C6-C2-C6 basic chemical structure. The best-known stilbene is resveratrol, present in grapes and blueberries, but other examples include α-viniferin, and astringin (Niesen et al., 2013). They can occur

naturally in the form of monomers, oligomers or with glycoside groups. Stilbenoids have antioxidant and antimicrobial properties and help fight inflammation and cancer in humans (Niesen et al., 2013). Tannins are phenolic compounds that bind to proteins, precipitate, or shrink them. They are known for their astringency and are large molecules with many hydroxyl and carboxyl groups (Ashok and Upadhyaya, 2012). Lignins are polymers that provide structure to the cell walls of plants. They are composed of three phenylpropane units with phenolic rings (guaiacyl, syringyl, and *p*-hydroxyphenyl) (Maphetu et al., 2022). Quinones have a benzoquinone as their base structure - a benzoic ring with two carbonyl groups on opposite sides. They can occur in multiple organisms besides plants, such as fungi, bacteria, and arthropods (Dulo et al., 2021).

2. Fermentation with food crop feedstocks

Fermentation has been used to produce food and beverages for thousands of years (Parapouli et al., 2020). This natural transformation involves the conversion of raw materials by living organisms into the desired end products. Traditionally, the yeast *S. cerevisiae* has held paramount importance in the field of fermentation, playing a pivotal role in the production of essential staples like bread and beer. In the modern era, *S. cerevisiae* has been applied to produce several compounds of interest at industrial scale, such as bioethanol. Through genetic manipulation of its genome, the ethanol productivities and tolerance to fermentation products have been successfully increased. Moreover, the introduction of heterologous genes (genes not native to *S. cerevisiae*) has enabled the yeast to metabolize sugars that it does not naturally consume, such as xylose from hydrolyzed lignocellulosic materials (Baptista et al., 2020). Additionally, genetic engineering has opened doors to the industrial production of other valuable molecules, including insulin, β-farnesene, and artemisinin. These advancements showcase the potential of *S. cerevisiae* as a versatile microorganism for biotechnological applications, including the synthesis of valuable compounds for various industries (Gomes et al., 2018; Kwak and Jin, 2017; Leavell et al., 2016; Meadows et al., 2016).

Farnesene, particularly β-farnesene, is a versatile molecule with a wide range of applications in industries such as cosmetics, surfactants, adjuvants, biofuels, and biogas (Benjamin et al., 2016; Carsanba et al., 2021; Jacobs et al., 2017). The production of β-farnesene can be derived from plant extraction. However, the low concentrations of β-

farnesene in plants render the economic feasibility of this process challenging (Liu et al., 2022). Additionally, efforts to pursue chemical synthesis of β-farnesene have been hindered by high production costs and low production efficiency, rendering large-scale production economically unfeasible (Yu et al., 2012). Thus, biosynthesis through microbial fermentation has emerged as the methodology for industrial production of β-farnesene. Currently, β-farnesene is produced by the company Amyris Inc, through the fermentation of sugarcane syrup in Brazil (Hill et al., 2020).

In a fermentation process, the carbon source stands as one of the primary ingredients, typically supplied by sugars such as glucose or sucrose. Currently, in industrial fermentations, plant sugars are mainly used to provide the carbon source for the microorganisms to grow and biosynthesize the desired compounds. Examples of these feedstocks are food crops or lignocellulosic materials. During the fermentation of food crop feedstocks, microorganisms possess the ability to directly consume sugars from materials like sugarcane juice and syrup. This grants them an advantage over the use of lignocellulosic materials, since the latter require the use of a pretreatment process to release the sugars from the cellulose or hemicellulose fractions (Jönsson and Martín, 2016; Ortega et al., 2021).

There are three modes of fermentation available for the industrial biosynthesis of value-added molecules. These include batch, fed-batch and continuous fermentations. In a batch fermentation, the carbon source is supplied in its entirety at the start of the incubation period. In a fed-batch fermentation, a relatively smaller amount of the carbon source is initially supplied at the beginning of the fermentation process, with additional quantities being continuously supplied over the course of the incubation period. Continuous fermentation involves the continuous addition of the carbon source over time, but simultaneously, fermentation broth is withdrawn at a corresponding rate, thereby maintaining a steady-state condition (Carsanba et al., 2021). In the industrial production of terpenoids, including β-farnesene, the fed-batch mode of fermentation is commonly employed since it combines the advantages of both batch and continuous fermentation modes (Mesquita et al., 2019; Zabed et al., 2014). This operating mode mitigates issues associated with high substrate concentrations present in a batch fermentation, by reducing inhibition arising from the Crabtree effect. Moreover, fed-batch fermentation offers other distinct advantages, including the ability to achieve high cell density, extended cell lifespan, maximum cell viability, and enhanced control over oxygen saturation. These factors

collectively contribute to increased product formation, leading to elevated yield and enhanced productivity (Zabed et al., 2014).

Food crop feedstocks typically serve as intermediate products derived from the sugar industry. For instance, sugarcane (*Saccharum officinarum* L.) is extensively utilized to produce sucrose (also called table sugar) in vast quantities. For sucrose production, the sugarcane is harvested and crushed, which results in the extraction of a juice that contains the sugars. Then, this juice is evaporated to concentrate the sugars and it is crystallized to remove impurities (Alarcón et al., 2020). Food crop feedstocks (juices and syrups) do not usually require crystallization or even the evaporation step. And, in fermentation, the direct use of sugarcane juice, syrup and molasse has been extensively studied (Carsanba et al., 2021; Sánchez and Cardona, 2008). A similar process occurs for other food crops, such as sugarbeet (*Beta vulgaris* L.), sorghum (*Sorghum bicolor* L.) and corn (*Zea mays* L.) (Joannis-Cassan et al., 2014; Renouf et al., 2008). In the case of starchy materials, like corn, there is also the need to hydrolyze the starch to obtain fermentable glucose (Sánchez and Cardona, 2008).

Sugarcane has been cultivated on a wide scale for many decades, primarily concentrated in regions with warm climates that suit the plant's environmental requirements. Brazil and India are the leading global producers of sugarcane, with Brazil growing 715.7 million tons and India growing 405.4 million tons in 2021, collectively accounting for 60 % of the world's sugarcane production that year (Figure 3). Consequently, the industrial production of sugarcane juices and syrups for fermentation predominantly takes place in these regions, and it is noteworthy that Amyris' biomanufacturing of β-farnesene is situated in Brazil. In this fermentation process, sugarcane syrup is supplied over time to bioreactors with the yeast *S. cerevisiae*.

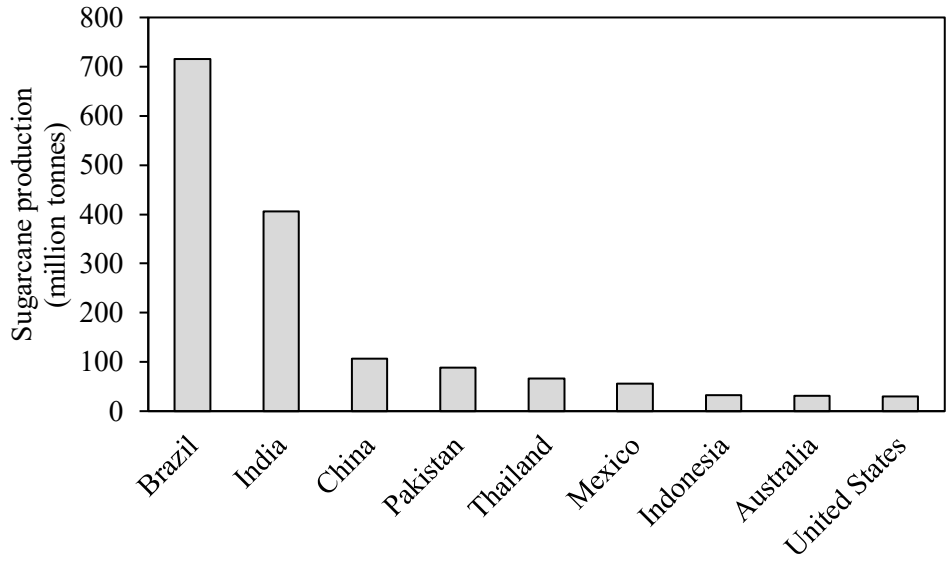

Figure 3. Sugarcane production by country during 2021 (source: FAOSTAT).

However, food crop feedstocks like sugarcane syrup contain more than just fermentable sugars within their composition. These feedstocks also encompass various substances that can influence the fermentation process, including organic acids, furans, and phenolic compounds. These additional components can have both positive and negative effects on the overall fermentation process (Adeboye et al., 2014; Cunha et al., 2018). The presence of these additional substances can increase the complexity of purifying the final product, leading to an increase in manufacturing costs (Yankov, 2022). Furthermore, substances like phenolic compounds can exert inhibitory effects on microorganisms, resulting in slowed growth and metabolism.

The antimicrobial activity of certain compounds contributes to a reduction in industrial efficiency, leading to escalated costs, and consequently, the fermentation consumes more resources and yields more expensive molecules. Conversely, most phenolic compounds possess remarkable antioxidant capacity. This attribute enhances the resistance of microorganisms to oxidative stress, enabling them to thrive and produce for extended periods (Wu et al., 2022).

The total phenolic content (TPC) in a sample is usually determined using the Folin-Ciocalteu methodology, a colorimetric assay employing gallic acid as the standard. Despite potential interferences, this method provides valuable estimations of the phenolic compounds' overall concentration. Studies have found the TPC of sugarcane juice to range

from approximately 160 to 460 mg of gallic acid equivalents per liter (mg GAE/L) (Brochier et al., 2016; Maurício Duarte-Almeida et al., 2006; Singh et al., 2015).

Conversely, specific phenolic compounds can be identified and quantified through chromatographic techniques such as high-performance liquid chromatography (HPLC) and liquid chromatography mass spectrometry (LC-MS) (Çayan et al., 2020; Oliveira et al., 2015). By employing these methods, the predominant phenolic compounds in sugarcane juice and syrup, which mainly comprise flavonoids and phenolic acids, have been identified. For instance, apigenin, luteolin and tricin are the more predominant flavonoids whereas hydroxycinnamic acid, caffeic acid, sinapic acid and chlorogenic acid are the main cinnamic acids (Duarte-Almeida et al., 2011; Maurício Duarte-Almeida et al., 2006; Singh et al., 2015). These studies revealed that sugarcane syrup contains approximately 200 mg/L of total phenolic content (TPC), with *p*-coumaric acid being the most abundant compound, present at 71.2 mg/L. Among the flavonoids, apigenin and tricin were found to be the most concentrated, at 29.6 mg/L and 27.6 mg/L, respectively.

Although numerous phenolic compounds have been identified and quantified in sugarcane juices and syrups, the impact of their combination on the fermentation process of *S. cerevisiae* remains largely unexplored. Hence, it becomes crucial to comprehend the interactions between these phenolic compounds from the feedstock and the yeast during the fermentation process. Understanding these interactions can provide valuable insights to optimize and enhance the fermentation process, ultimately leading to improved efficiency and production outcomes.

3. Effects of phenolic compounds in microorganisms

3.1.Antioxidant activity

Every aerobic cell, whether it is in a multicellular or unicellular organism, is subjected to oxygen, which is an oxidant. Oxygen, in a molecular state, is relatively unreactive. However, organic compounds and their structures are thermodynamically unstable in an oxygen-containing atmosphere. This leads to the formation and accumulation of free radicals and other reactive oxygen species (ROS) (Bednarska et al., 2008). These molecules continuously accumulate during the lifetime of a cell, causing oxidative stress, and are ultimately correlated with aging (Sadowska-Bartosz and Bartosz, 2014). Oxidative

damage can be found on a molecular level as oxidized lipids, proteins, or DNA. Many types of ROS have been shown to induce a certain type of damage in the subjected cells. Examples of ROS are: the radicals superoxide ($O_2^{\cdot -}$), hydroxyl ($OH^{\cdot}$), alkoxyl ($RO^{\cdot}$), peroxyl ($RO_2^{\cdot}$), nitric oxide ($NO^{\cdot}$), nitrogen dioxide ($NO_2^{\cdot}$) and the non-radicals hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl) and singlet oxygen (Mehta et al., 2015).

Oxidative stress can play a critical role in yeast fermentation processes, representing a significant area of investigation in the field of yeast physiology and metabolism. During aerobic fermentation, yeast cells encounter various stressors that can disrupt cellular redox homeostasis, leading to the generation of ROS and subsequent oxidative damage (Sadowska-Bartosz and Bartosz, 2014). Therefore, antioxidants play a pivotal role in mitigating the detrimental effects of oxidative stress in yeast fermentation processes.

Protection against damage induced by oxidative stress can be achieved through three mechanisms: a preventive, a chain-breaking or a repair mechanism (Murkovic, 2003). The preventive method involves the suppression of free-radical formation by antioxidants, which may include inhibiting enzymes responsible for catalyzing the formation of these harmful radicals. The chain-breaking method operates by interrupting oxidation chain reactions through various manners. This includes quenching singlet oxygen, chelating metals to convert pro-oxidative metal ions (e.g., iron and copper) into stable products, and inhibiting pro-oxidative enzymes like lipoxygenases. These actions collectively contribute to the prevention of oxidative damage and help maintain cellular integrity. The repair method involves the regeneration of ROS back into their non-reactive state (Murkovic, 2003).

Singlet oxygen quenching is essential to counteract the naturally occurring reactions, in which a photosensitizer is illuminated with a light with a specific wavelength and is then raised into an excited state. Then, the photosensitizer returns to the grounded state by transferring the excitation energy to the oxygen molecule, creating singlet oxygen. This molecule can cause damage to the cells by reallocating excitation energy to a co-reactant, resulting in detrimental effects on cellular components (Ďuračková, 2014; Hur et al., 2014).

Transition metal chelation is another mechanism utilized by the cells to avoid oxidative stress. Naturally, free ions of metals, such as iron and copper, react with H_2O_2 generating ROS, as described by the Fenton reaction. However, due to the presence of chelators, the metals relate to ligands, inhibiting this reaction and lowering the reactivity of these molecules (Ďuračková, 2014; Hur et al., 2014).

Most antioxidants are polyphenolic compounds, serving as versatile agents in various ways. They act as reducing agents, effectively terminating free radicals, chelate metals, quench singlet oxygen, and donate hydrogen to neutralize harmful reactive species. This multifaceted nature of polyphenolic antioxidants makes them highly effective in combating oxidative stress and protecting cells from damage (Hur et al., 2014). Monophenols are less effective than polyphenols due to the absence of the second hydroxyl group at either ortho or para position, which considerably increases the antioxidant activity. For instance, protocatechuic acid, a polyphenol, is a more effective antioxidant than its respective monophenol, *p*-hydroxybenzoic acid. However, with the addition of one or two methoxylic substituents, the antioxidant activity of monophenols can be further improved (Merkl et al., 2010).

Polyphenols, such as flavonoids, are excellent antioxidants because they have two hydroxyl groups in their B ring and a galloyl ester in the C ring, which are also important structures in metal ion chelation (Hur et al., 2014). For instance, trolox and quercetin show great antioxidant activities. The antioxidant activity of flavonoids also comes from the presence of an *o*-diphenolic group (a 2–3 double bond conjugated with the 4-oxo function and hydroxyl groups in positions 3 and 5) (Hur et al., 2014). Flavonoids can effectively scavenge hydroxyl and peroxyl radicals, constitute complexes with metals, and inhibit lipid oxidation initiated by metals.

Moreover, phenolic acids have also been recognized for their significant antioxidant activity, with protocatechuic acid being identified as the most potent simple phenolic acid in terms of antioxidant capacity, followed by caffeic and chlorogenic acids (Terpinc and Abramovič, 2010). Indeed, studies have revealed that protocatechuic acid exhibits remarkable potency in inhibiting free radical formation (IC_{50}), surpassing the inhibitory activity of certain flavonoids (Chen et al., 2013; Erkan et al., 2011). This highlights the significant antioxidant potential of protocatechuic acid, making it a valuable candidate for oxidative stress mitigation and cellular protection (Table 1).

Table 1. Antioxidant activity of phenolic compounds assessed by DPPH and ORAC methodologies. IC_{50} values are presented, indicating the concentration required to inhibit 50 % of free radical formation. DPPH: 2,2-diphenyl-1-picrylhydrazyl; ORAC: oxygen radical absorbance capacity.

Phenolic compound	Method	IC_{50} (mM)	Reference
Chlorogenic acid	DPPH	0.025	Terpinc and Abramovič, 2010
	DPPH	0.100	Erkan et al., 2011
	ORAC	0.028	Chen et al., 2013
Protocatechuic acid	DPPH	0.022	Terpinc and Abramovič, 2010
	ORAC	0.017	Chen et al., 2013
Caffeic acid	DPPH	0.026	Terpinc and Abramovič, 2010
	DPPH	0.069	Erkan et al., 2011
	ORAC	0.024	Chen et al., 2013
p-Coumaric acid	DPPH	2.550	Terpinc and Abramovič, 2010
	DPPH	0.641	Erkan et al., 2011
Ferulic acid	DPPH	0.049	Terpinc and Abramovič, 2010
	DPPH	0.255	Erkan et al., 2011
(-)-Epicatechin	ORAC	0.020	Chen et al., 2013
Quercetin	DPPH	0.045	Erkan et al., 2011
	ORAC	0.013	Chen et al., 2013
Apigenin	DPPH	1.474	Erkan et al., 2011

In the context of high cell density industrial fermentations, the provision of a constant and elevated oxygen supply poses a significant challenge (Li et al., 2011). It has been found that increasing oxygen concentration leads to more ROS formation, which leads to cell metabolic responses through enzymatic production of superoxide dismutase, catalase and NADH dehydrogenase (Bai et al., 2003; O'Donnell et al., 2007). Consequently, cultures with high oxygen supply have decreased ATP generation and carbon uptake rates (O'Donnell et al., 2007).

In fact, there is compelling evidence to support the notion that reducing oxidative stress during fermentation not only reduces the enzymatic and non-enzymatic oxidative cellular response, but also enhances fermentation productivity. This has been demonstrated through the incorporation of antioxidant polypeptides as supplements in the culture medium during the malic acid production process (Wu et al., 2022). Hence, incorporating additional antioxidants, such as phenolic compounds, into the culture medium can lead to similar outcomes in terms of mitigating oxidative stress. This can attenuate both enzymatic and non-

enzymatic oxidative responses in cells, thereby potentially enhancing fermentation productivity. By providing a protective environment against oxidative damage, these antioxidants can positively impact the overall performance and efficiency of the fermentation process.

3.2.Pro-oxidant activity

Maintaining a redox balance within a yeast cell is of utmost importance for its overall cellular health and functionality. Proper redox regulation ensures the preservation of essential cellular processes, including metabolism, growth, and defense against oxidative stress. Besides antioxidant activity, some phenolic compounds have been associated with pro-oxidant activities. This phenomenon has the potential to disrupt the cellular redox balance and consequently impact yeast fermentation processes (Castaneda-Arriaga et al., 2018). For instance, hydroxybenzoic acid, a monophenol, has been shown to have more pro-oxidant than antioxidant activity (Castaneda-Arriaga et al., 2018). This has been shown for *p*-hydroxybenzoic acid, as well as for its polyphenol derivatives (dihydroxybenzoic acids) containing two hydroxyl groups in the 2-6, 3-4 and 3-5 positions (Castaneda-Arriaga et al., 2018; Kalinowska et al., 2021).

Furthermore, it is noteworthy that certain flavonoids can also exhibit pro-oxidant activity when present in excessive concentrations or in the presence of reduced metals (Sotler et al., 2019). For example, quercetin and kaempferol can cause DNA damage and lipid peroxidation when exposed to transition metals. In the presence of iron or copper, phenolic compounds can undergo redox cycling, resulting in the formation of phenolic radicals (Sotler et al., 2019). These radicals possess the capability to disrupt cellular integrity, potentially leading to cellular damage. Furthermore, studies have demonstrated that catechin and epigallocatechin gallate (EGCG) can induce a cellular oxidative stress response in *S. cerevisiae*, indicating their impact on cellular health and redox balance (Kim et al., 2006).

3.3.Inhibition of microbial growth and toxicity

Phenolic compounds can significantly impact the yeast fermentation process through their antimicrobial activity, which arises from their ability to damage the cell membrane.

The diminished integrity of the cell membrane disrupts its essential dual function as a selective barrier, hindering the controlled passage of molecules, and as an enzyme matrix, compromising the organization and functionality of enzymes within the membrane structure (Adeboye et al., 2014; Kim et al., 2013; Zhang et al., 2016). High concentrations of certain phenolic compounds can become toxic to the yeast and cause cell death (Fosso-Kankeu et al., 2015; Gu et al., 2015; Teymennet-Ramírez et al., 2022). Some studies reported that the modifications in the composition of the cell membrane are related to the release of saturated fatty acids from the cell membrane (Gu et al., 2015; Wang et al., 2015; Xie et al., 2016). The interaction of phenolic compounds with the lipids and membrane-embedded proteins causes the change in the cell membrane. In addition, a permeable cell membrane produces an electrochemical imbalance caused by an undesired efflux of K^+ ions (Gu et al., 2015; Wang et al., 2015; Xie et al., 2016). Furthermore, high cell membrane permeability has been shown to inhibit the growth of the yeast *S. cerevisiae* (Galant et al., 2017; López et al., 2021; Thevissen et al., 1999).

It has been found that reduced molecule polarity of phenolic compounds increases the antimicrobial activity. Thus, alkyl esters of phenolic acids have increased antimicrobial activities than pure acids. Lower polarity increases hydrophobicity, which improves solubility in oil and facilitates the access of the phenolic compounds to the lipophilic cell wall of the targeted microorganisms (Almeida et al., 2007; Gu et al., 2015; Xie et al., 2016). The antimicrobial effect of these phenolic compounds also increases with the length of the alkyl chain. For example, butyl esters of phenolic acids inhibit the growth of *S. cerevisiae* more than methyl esters (Guil-Guerrero et al., 2016; Merkl et al., 2010). Moreover, according to the study conducted by Pizzolitto et al., 2015, vanillin, a phenolic compound known for its high hydrophobicity, exhibited greater activity against the fungus *Aspergillus parasiticus* when compared to m-cresol and phenol.

Phenolic acids, aldehydes, alcohols, and ketones have been shown to exert inhibitory effects on cellular activity. Multiple studies have reported the inhibitory impact of phenolic compounds on *S. cerevisiae*, with lower molecular weight monophenols exhibiting greater inhibitory effects compared to polyphenols. Examples of inhibitory monophenols are 4-hydroxybenzoic acid, syringaldehyde, syringic acid, coniferyl aldehydes, *p*-coumaric acid, ferulic acid and vanillin (Almeida et al., 2007; Kim et al., 2013; Lin et al., 2015; Wang et al., 2017). Furthermore, the toxicity of phenolic compounds can be influenced by the

presence of a hydroxyl group (-OH) in the ortho position (as depicted in Figure 4 with vanillin). However, toxicity is not observed when the hydroxyl group is situated in the para or meta positions (Almeida et al., 2007). Moreover, the same condition can form an intramolecular hydrogen bond within phenolic aldehydes, contributing to membrane permeability and toxicity (Xie et al., 2016).

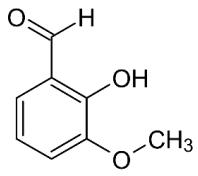

Figure 4. Vanillin structure with hydroxyl group (-OH) in the ortho position.

The inhibitory effect of phenolic compounds is a result of the combination of the functional groups (such as methoxy and hydroxyl groups) and the presence of unsaturated bonds in the structure of a phenolic compound (Adeboye et al., 2014). For instance, ferulic acid and *p*-coumaric acid possess a double bond between two carbon atoms, which links the carboxylic group to the aromatic ring. In contrast, syringic acid lacks this double bond in its structure (Figure 4). The toxicity limits for these compounds are as follows: ferulic acid has a limit of 1.8 mM, *p*-coumaric acid has a limit of 9.7 mM, and syringic acid exhibits a higher limit, exceeding 22 mM (Adeboye et al., 2014). Ferulic acid is the most toxic of the three molecules, which contains the hydroxyl group in the ortho position and the double bond linking the carboxyl group to the aromatic ring (Figure 5). Furthermore, the inhibitory effect of the same molecule on a specific microorganism can change with its concentration, displaying a dose-dependent inhibition (Cola et al., 2020).

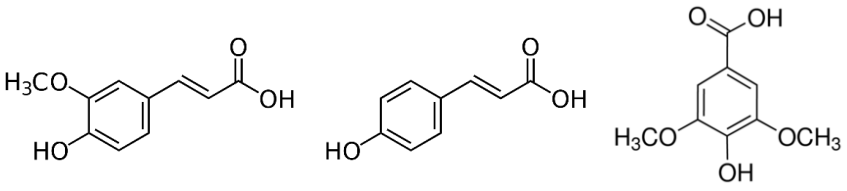

Figure 5. Ferulic acid (A), *p*-coumaric acid (B) and syringic acid (C) structures.

In addition, Adeboye et al., 2014 reported coniferyl aldehyde as the most toxic phenolic compound, with total inhibition of *S. cerevisiae* growth at 1.4 mM (Table 2). This compound also contains both the hydroxyl group in the ortho position and the double bond linking the carboxyl group to the aromatic ring (Figure 6). On the other hand, Gu et al., 2015 reported that cinnamic acid completely inhibits growth at lower concentrations (1.0 mM).

Table 2. Inhibitory effects of phenolic compounds on the yeast *S. cerevisiae*.

Phenolic compound	Concentration (mM)	Reported effects	Reference
Coniferyl aldehyde	1.4	Decreased growth rate by 80 %	(Adeboye et al., 2015)
	6.7	Decreased growth rate by 91 %	(Gu et al., 2015)
p-Coumaric acid	9.7	Decreased growth rate by 80 %	(Adeboye et al., 2015)
	9.7	Total growth inhibition	(Cola et al., 2020)
	7.3	Decreased growth rate by 96 %	(Gu et al., 2015)
Phenol	30	Total growth inhibition	(Li et al., 2017)
Acetosyringone	5.1	Decreased growth rate by 22 %	(Colombi et al., 2018)
Syringaldehyde	5.5	Decreased growth rate by 56 %	(Colombi et al., 2018)
	6.6	Decreased growth rate by 5 %	(Gu et al., 2015)
	30	Delayed cell growth by 24 h	(Li et al., 2017)
Syringic acid	5.0	Decreased growth rate by 33 %	(Colombi et al., 2018)
Vanillin	6.5	Decreased growth rate by 63 %	(Colombi et al., 2018)
	7.9	Decreased growth rate by 71 %	(Gu et al., 2015)
	20	Total growth inhibition	(Li et al., 2017)
Vanillic acid	11.9	Decreased growth rate by 44 %	(Gu et al., 2015)
	10	Total growth inhibition	(Merkl et al., 2010)
Ferulic acid	>20	Total growth inhibition	(Merkl et al., 2010)
	5.1	Decreased growth rate by 76 %	(Gu et al., 2015)
2-Furoic acid	10.7	Decreased growth rate by 96 %	(Gu et al., 2015)
Caffeic acid	20	Total growth inhibition	(Merkl et al., 2010)
p-Hydroxybenzoic acid	20	Total growth inhibition	(Merkl et al., 2010)
p-Hydroxybenzaldehyde	9.8	Decreased growth rate by 67 %	(Gu et al., 2015)
Protocatechuic acid	>20	Total growth inhibition	(Merkl et al., 2010)
Cinnamic acid	1.0	Total growth inhibition	(Gu et al., 2015)
Guaiacol	8	Decreased growth rate by 17 %	(Gu et al., 2015)

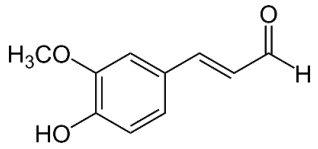

Figure 6. Coniferyl aldehyde structure.

Besides the individual effect of each molecule, mixtures of phenolic compounds can elicit negative synergistic effects on *S. cerevisiae*. When combined, phenolic compounds have the capability to amplify their collective antimicrobial activities, surpassing the sum of the activities exhibited by the individual phenolic compounds. This has been demonstrated regarding the inhibitory activity of certain phenolic compounds, such as gallic acid, caffeic acid, vanillic acid, *p*-hydroxybenzoic acid, syringic acid and dihydroxybenzaldehyde (Ansari et al., 2013; Gu et al., 2019; Lima et al., 2016). When multiple phenolic compounds were present simultaneously, their inhibition was amplified. Furthermore, when other inhibitors such as acetic acid or furans are present in the culture medium, they can also synergistically interact with phenolic compounds, resulting in combined effects that exceed the additive impact of these compounds individually (Cunha et al., 2018; Ding et al., 2011; Gu et al., 2019).

On the other hand, the antimicrobial effect of phenolic compounds varies with the scale of the fermentation process. Some studies have demonstrated that the outcomes of antimicrobial assays can differ depending on the experimental systems, such as microplates, shake-flasks, or bioreactors (Adeboye et al., 2015). The observed difference between scales suggests that fermentations conducted on larger scales may display reduced antimicrobial effects compared to those carried out on smaller scales. For example, at a smaller scale, the growth of *S. cerevisiae* was completely inhibited using just 1.4 mM of coniferyl aldehyde. However, in bioreactor fermentations, there was no decrease in growth rate with 1.1 mM of coniferyl aldehyde. In fact, the growth rate increased 11 % compared to the control. Additionally, when 9.7 mM of *p*-coumaric acid was used in shake flask fermentations, the growth of the yeast was completely inhibited, however the same concentration in the bioreactor experiments resulted in only 22 % of decrease in the growth rate (Adeboye et al., 2015, 2014). This observation has been attributed to improved agitation, aeration, and pH control in large-scale fermentations, as well as the presence of higher cell density, which

collectively contribute to a less favorable environment for the manifestation of antimicrobial effects by phenolic compounds (Adeboye et al., 2015). Culture aeration has been shown to increase the conversion rate of phenolic compounds (He et al., 2016). In addition, fermentations conducted at high cell densities result in increased cell survival, elevated conversion rates of inhibitors, and subsequent decrease in their inhibitory effects (Almeida et al., 2007; Guo and Olsson, 2016; Soares et al., 2020). Thus, high cell density can reduce the duration of the lag phase, shorten the fermentation time, and increase productivity.

4. Strategies for mitigating phenolic compounds' inhibition in fermentation

The presence of phenolic compounds at inhibitory concentrations can lead to inefficient fermentation processes. Consequently, it becomes imperative to adopt strategies aimed at mitigating the inhibition of growth and production caused by these compounds. Fortunately, several viable approaches are available for this purpose. Phenolic content can be effectively removed from the feedstock through adsorption onto specific materials. Examples of such materials include activated charcoal and bead resins (Ahmaruzzaman, 2008; Bazrafshan et al., 2016; Rahmanian et al., 2014; Raza et al., 2019). For instance, activated charcoal has been applied in the removal of phenolic compounds from corn stover, resulting in a detoxified feedstock that allowed for a successful fermentation of itaconic acid, with 33.6 g/L of final concentration (Liu et al., 2020). On the other hand, Amberlite XAD-4 resin has been used to remove phenolic compounds from oil pam frond juice, a material later used to increase the fermentation production of biobutanol by 3-fold (Abubakar et al., 2023).

Other strategies available to remove phenolic compounds from feedstocks include ozone treatment and membrane filtration (Rahmanian et al., 2014; Raza et al., 2019). For instance, ozone has been used to remove phenolic compounds from palm oil mill effluent, improving the fermentation process of methane by improving the yield by 48 % (Krishnan et al., 2023). In addition, membrane filtration has improved bioethanol production from rice straw, with 1.7-fold more final ethanol concentration (Pan et al., 2019).

Another methodology for reducing the phenolic content of the feedstock is the use of organic solvents. This chemical extraction is possible because the non-polar phenolic compounds are more soluble in non-polar solvents, such as ethanol and methanol

(Rahmanian et al., 2014). However, these methods are usually applied for the recovery of phenolic compounds for other applications.

On the other hand, biological strategies can also be employed for the removal of phenolic compounds. This can be achieved by utilizing microorganisms capable of converting the phenolic compounds into other non-inhibitory compounds. For instance, *S. cerevisiae* can naturally convert certain phenolic aldehydes into alcohols. Coniferyl aldehyde, for example, undergoes conversion to coniferyl alcohol and dihydroconiferyl alcohol, a process likely facilitated by an oxidoreductase enzyme (Adeboye et al., 2015; Gu et al., 2015; Jönsson and Martín, 2016). In addition, phenolic conversion can be directly performed with the use of the enzymes, in immobilized form (Raza et al., 2019).

Another effective strategy to minimize phenolic inhibition involves optimizing the fermentation process. Rather than using batch fermentation, where all feedstock is added at the beginning, a fed-batch fermentation approach can be employed. This method allows for the gradual addition of feedstock during the process, leading to a reduced initial concentration of phenolic compounds. As a result, the inhibitory effects of these compounds are decreased, enhancing the overall fermentation efficiency (Rawoof et al., 2020). Secondly, the use of high cell density cultures to initiate the fermentation process can be advantageous, as they exhibit greater resistance to the toxicity caused by phenolic compounds and possess the ability to convert them into non-toxic molecules. As a result, shorter lag phases are observed, and the overall productivity of the fermentation is significantly increased (Almeida et al., 2007; Guo and Olsson, 2016; Soares et al., 2020).

In addition to reducing the concentration of phenolic compounds in the culture medium, implementing strategies to enhance inhibition tolerance during fermentation can be highly beneficial. This can be achieved by utilizing industrial strains of microorganisms, particularly *S. cerevisiae*, that have been specially adapted to thrive in the presence of inhibitory compounds. These industrial strains have demonstrated superior performance compared to laboratory strains due to their increased tolerance to the stress conditions prevalent in industrial fermentation environments. As a result, these robust industrial strains can effectively overcome inhibitory challenges and lead to more efficient and successful fermentation processes (Cola et al., 2020).

Apart from natural mechanisms that *S. cerevisiae* contains to tolerate phenolic compounds, efforts have been made to develop strains that possess increased tolerance to

some phenolic compounds by identifying and overexpressing endogenous genes, or expressing heterologous genes, that code for enzymes of interest (Adeboye et al., 2017, Jönsson et al., 2013). These genetic engineering strategies increase the concentrations on detoxifying enzymes in the cytoplasm, which results in the conversion of phenolic compounds into less toxic metabolites.

Overexpression of some genes in *S. cerevisiae* has been reported, such as for genes coding the enzymes Yap1p, Atr1p, and Flr1p. These modifications contributed to the cellular defense against the naturally occurring compound coniferyl aldehyde (Sundström et al., 2010). Another example is phenylacrylic acid decarboxylase (PAD), which is an aromatic acid decarboxylase that converts cinnamic, *p*-coumaric and ferulic acids. Some authors have demonstrated that the overexpression of this enzyme can result in improved growth parameters (Adeboye et al., 2015; Almeida et al., 2007; Gu et al., 2015; Larsson et al., 2001; Richard et al., 2015). Additionally, it has been shown that overexpressing the genes ALD5, ATF1 and ATF2 (alcohol acetyltransferases) in *S. cerevisiae* was a feasible strategy to increase resistance to coniferyl aldehyde, ferulic acid and *p*-coumaric (Adeboye et al., 2017). Lastly, the conversion of vanillin has been extensively studied. It was found that overexpression of many genes has effects on increasing vanillin resistance and detoxification in *S. cerevisiae* - ADH6, YNL134C, YJR09W, ALD6, ZWF1, MCH2, SNG1, GPH1 and TMA10 (Ďuračková, 2014; García-López et al., 2010; Grey et al., 1995; Park and Kim, 2014; Wang et al., 2016; Wohler Sunnarborg et al., 2001).

Regarding the addition of heterologous expression, some enzymes, such as phenol oxidase laccase and lignin peroxidase from polypore mushroom *Trametes versicolor*, have been reported to increase sugar consumption and ethanol production rates in a fermentation of spruce hydrolysate (Larsson et al., 2001).

Furthermore, it has been demonstrated that the ALD5 gene, which encodes a mitochondrial aldehyde dehydrogenase, plays a crucial role in the conversion of coniferyl aldehyde in *S. cerevisiae* (Adeboye et al., 2017).

5. Conclusions

Food crop feedstocks, such as the juices and syrups from sugarcane, are supplied to fermentation to produce multiple valuable molecules. Their phenolic content is mainly

composed of phenolic acids and flavonoids. The most predominant flavonoids in sugarcane juices and syrups are apigenin, luteolin and tricin and they have great antioxidant activity. On the other hand, the most concentrated phenolic acids are hydroxycinnamic, caffeic, sinapic, chlorogenic and *p*-coumaric acids. Phenolic acids can present both antioxidant and antimicrobial activities. Antioxidant activity provides protection against oxidative stress present in *S. cerevisiae* fermentations, while antimicrobial activity hinders the fermentation process by inhibiting enzyme activity, and the growth and production rates. In addition, the impact of phenolic compounds on the yeast depends on its concentration.

Multiple strategies are available to mitigate the inhibition of phenolic compounds from feedstocks in fermentation. These strategies have successfully been used to remove the phenolic compounds and improve fermentation processes, and include the adsorption of phenolic compounds into activated charcoal or bead resins, ozone treatment and membrane filtration. On the other hand, the use of high cell density cultures in the beginning of the fermentation and the application of a fed-batch mode during the fermentation also decrease the inhibition of phenolic compounds. In addition, genetic engineering is also a viable strategy to increase the tolerance of *S. cerevisiae* to phenolic compounds.

Juices and syrups from sugarcane contain low concentrations of phenolic compounds compared to lignocellulosic materials. However, it is not yet understood if these levels of phenolic compounds can produce a harmful or a beneficial effect on the fermentation. Therefore, further studies are required to comprehensively evaluate the actual impact of phenolic compounds in the fermentation utilizing food crops feedstocks. For instance, it is necessary to characterize and evaluate the phenolic compounds during fermentation processes. This would allow the identification of the specific phenolic compounds present in the fermentation broth and enable quantification of their respective concentrations. Additionally, conducting fermentation using a feedstock with reduced phenolic compounds would provide valuable insights into their influence on yeast physiology and metabolism. Furthermore, the strategic supplementation of phenolic compounds during yeast fermentation may also present a viable avenue for assessing if there are benefits in increasing the concentrations of antioxidant phenolic compounds on the fermentation process.

References

Abubakar, A., Salleh, M.B.M., Yahya, A.B., Shiong, C.C., Mohamad, S.E., Abd-Aziz, S.B., Hussin, H., 2023. Phenolic Compound Removal Technique For Efficient Biobutanol Production Using Oil Palm Fronds Hydrolysate. Journal of Renewable Energy and Environment. https://doi.org/10.30501/JREE.2023.374488.1512

Adeboye, P.T., Bettiga, M., Aldaeus, F., Larsson, P.T., Olsson, L., 2015. Catabolism of coniferyl aldehyde, ferulic acid and p-coumaric acid by *Saccharomyces cerevisiae* yields less toxic products. Microb Cell Fact 14, 149. https://doi.org/10.1186/s12934-015-0338-x

Adeboye, P.T., Bettiga, M., Olsson, L., 2017. ALD5, PAD1, ATF1 and ATF2 facilitate the catabolism of coniferyl aldehyde, ferulic acid and p-coumaric acid in *Saccharomyces cerevisiae*. Sci Rep 7, 42635. https://doi.org/10.1038/srep42635

Adeboye, P.T., Bettiga, M., Olsson, L., 2014. The chemical nature of phenolic compounds determines their toxicity and induces distinct physiological responses in *Saccharomyces cerevisiae* in lignocellulose hydrolysates. AMB Express 4, 46. https://doi.org/10.1186/s13568-014-0046-7

Ahmaruzzaman, M., 2008. Adsorption of phenolic compounds on low-cost adsorbents: A review. Adv Colloid Interface Sci 143, 48–67. https://doi.org/10.1016/J.CIS.2008.07.002

Alarcón, Á.L., Orjuela, A., Narváez, P.C., Camacho, E.C., 2020. Thermal and Rheological Properties of Juices and Syrups during Non-centrifugal Sugar Cane (Jaggery) Production. Food and Bioproducts Processing 121, 76–90. https://doi.org/10.1016/J.FBP.2020.01.016

Albuquerque, B.R., Heleno, S.A. 8., Oliveira, M.B.P.P., Barros, L., Ferreira, I.C.F.R., 2021. Phenolic compounds: current industrial applications, limitations and future challenges. Food Funct 12, 14–29. https://doi.org/10.1039/D0FO02324H

Almeida, J.R.M., Modig, T., Petersson, A., Hähn-Hägerdal, B., Lidén, G., Gorwa-Grauslund, M.F., 2007. Increased tolerance and conversion of inhibitors in lignocellulosic hydrolysates by *Saccharomyces cerevisiae*. Journal of Chemical Technology & Biotechnology 82, 340–349. https://doi.org/10.1002/jctb.1676

Ansari, M., Anurag, A., Fatima, Z., Hameed, S., 2013. Natural Phenolic Compounds: A Potential Antifungal Agent.

Ashok*, P.K., Upadhyaya, K., 2012. Tannins are Astringent. J Pharmacogn Phytochem 1, 45–50.

Bai, Z., Harvey, L.M., McNeil, B., 2003. Physiological responses of chemostat cultures of *Aspergillus niger* (B1-D) to simulated and actual oxidative stress. Biotechnol Bioeng 82, 691–701. https://doi.org/10.1002/BIT.10614

Baptista, S.L., Carvalho, L.C., Romaní, A., Domingues, L., 2020. Development of a sustainable bioprocess based on green technologies for xylitol production from corn cob. Ind Crops Prod 156, 112867. https://doi.org/https://doi.org/10.1016/j.indcrop.2020.112867

Bazrafshan, E., Amirian, P., Mahvi, A.H., Ansari-Moghaddam, A., 2016. Application of adsorption process for phenolic compounds removal from aqueous environments: A systematic review. Global Nest Journal 18, 146–163. https://doi.org/10.30955/GNJ.001709

Bednarska, S., Leroy, P., Zagulski, M., Bartosz, G., 2008. Efficacy of antioxidants in the yeast *Saccharomyces cerevisiae* correlates with their effects on protein thiols. Biochimie 90, 1476–1485. https://doi.org/10.1016/j.biochi.2008.05.013

Benjamin, K.R., Silva, I.R., Cherubim, J.P., McPhee, D., Paddon, C.J., 2016. Developing Commercial Production of Semi-Synthetic Artemisinin, and of β-Farnesene, an Isoprenoid Produced by Fermentation of Brazilian Sugar. J Braz Chem Soc 27, 1339–1345. https://doi.org/10.5935/0103-5053.20160119

Brochier, B., Mercali, G.D., Marczak, L.D.F., 2016. Influence of moderate electric field on inactivation kinetics of peroxidase and polyphenol oxidase and on phenolic compounds of sugarcane juice treated by ohmic heating. LWT 74, 396–403. https://doi.org/10.1016/J.LWT.2016.08.001

Carsanba, E., Pintado, M., Oliveira, C., 2021. Fermentation Strategies for Production of Pharmaceutical Terpenoids in Engineered Yeast. Pharmaceuticals 14. https://doi.org/10.3390/PH14040295

Castaneda-Arriaga, R., Pérez-González, A., Reina, M., Alvarez-Idaboy, J.R., Galano, A., 2018. Comprehensive Investigation of the Antioxidant and Pro-oxidant Effects of Phenolic Compounds: A Double-Edged Sword in the Context of Oxidative Stress? Journal of Physical Chemistry B 122, 6198–6214. https://doi.org/10.1021/ACS.JPCB.8B03500/ASSET/IMAGES/LARGE/JP-2018-03500V_0010.JPEG

Çayan, F., Deveci, E., Tel-Çayan, G., Duru, M.E., 2020. Identification and quantification of phenolic acid compounds of twenty-six mushrooms by HPLC–DAD. Journal of Food Measurement and Characterization 14, 1690–1698. https://doi.org/10.1007/S11694-020-00417-0/TABLES/4

Chen, C.Y., Li, H., Yuan, Y.N., Dai, H.Q., Yang, B., 2013. Antioxidant activity and components of a traditional Chinese medicine formula consisting of *Crataegus pinnatifida* and *Salvia miltiorrhiza*. BMC Complement Altern Med 13. https://doi.org/10.1186/1472-6882-13-99

Cola, P., Procópio, D.P., Alves, A.T. de C., Carnevalli, L.R., Sampaio, I.V., da Costa, B.L.V., Basso, T.O., 2020. Differential effects of major inhibitory compounds from sugarcane-based lignocellulosic hydrolysates on the physiology of yeast strains and lactic acid bacteria. Biotechnol Lett 42, 571–582. https://doi.org/10.1007/s10529-020-02803-6

Colombi, B.L., Zanoni, P.R.S., Tavares, L.B.B., 2018. Effect of phenolic compounds on bioconversion of glucose to ethanol by yeast *Saccharomyces cerevisiae* PE-2. Can J Chem Eng 96, 1444–1450. https://doi.org/10.1002/cjce.23114

Cunha, J.T., Romaní, A., Costa, C.E., Sá-Correia, I., Domingues, L., 2018. Molecular and physiological basis of *Saccharomyces cerevisiae* tolerance to adverse lignocellulose-based process conditions. Applied Microbiology and Biotechnology 2018 103:1 103, 159–175. https://doi.org/10.1007/S00253-018-9478-3

de la Rosa, L.A., Moreno-Escamilla, J.O., Rodrigo-García, J., Alvarez-Parrilla, E., 2019. Phenolic Compounds. Postharvest Physiology and Biochemistry of Fruits and Vegetables 253–271. https://doi.org/10.1016/B978-0-12-813278-4.00012-9

Dias, R., Oliveira, H., Fernandes, I., Simal-Gandara, J., Perez-Gregorio, R., 2020. Recent advances in extracting phenolic compounds from food and their use in disease

prevention and as cosmetics. https://doi.org/10.1080/10408398.2020.1754162 61, 1130–1151. https://doi.org/10.1080/10408398.2020.1754162

Ding, M.Z., Wang, X., Yang, Y., Yuan, Y.J., 2011. Metabolomic Study of Interactive Effects of Phenol, Furfural, and Acetic Acid on *Saccharomyces cerevisiae*. https://home.liebertpub.com/omi 15, 647–653. https://doi.org/10.1089/OMI.2011.0003

Duarte-Almeida, J.M., Salatino, A., Genovese, M.I., Lajolo, F.M., 2011. Phenolic composition and antioxidant activity of culms and sugarcane (*Saccharum officinarum* L.) products. Food Chem 125, 660–664. https://doi.org/10.1016/j.foodchem.2010.09.059

Dulo, B., Phan, K., Githaiga, J., Raes, K., De Meester, S., 2021. Natural Quinone Dyes: A Review on Structure, Extraction Techniques, Analysis and Application Potential. Waste and Biomass Valorization 2021 12:12 12, 6339–6374. https://doi.org/10.1007/S12649-021-01443-9

Ďuračková, Z., 2014. Free Radicals and Antioxidants for Non-Experts, in: Laher, I. (Ed.), Systems Biology of Free Radicals and Antioxidants. Springer Berlin Heidelberg, Berlin, Heidelberg, pp. 3–38. https://doi.org/10.1007/978-3-642-30018-9_2

Dwivedi, M.K., Sonter, S., Mishra, S., Patel, D.K., Singh, P.K., 2020. Antioxidant, antibacterial activity, and phytochemical characterization of *Carica papaya* flowers. Beni Suef Univ J Basic Appl Sci 9, 1–11. https://doi.org/10.1186/S43088-020-00048-W/FIGURES/3

Erkan, N., Akgonen, S., Ovat, S., Goksel, G., Ayranci, E., 2011. Phenolic compounds profile and antioxidant activity of *Dorystoechas hastata* L. Boiss et Heldr. Food Research International 44, 3013–3020. https://doi.org/10.1016/J.FOODRES.2011.07.015

FAOSTAT [WWW Document], n.d. URL https://www.fao.org/faostat/en/#data/QCL (accessed 8.4.23).

Galant, L.S., Braga, M.M., de Souza, D., de Bem, A.F., Sancineto, L., Santi, C., da Rocha, J.B.T., 2017. Induction of reactive oxygen species by diphenyl diselenide is preceded by changes in cell morphology and permeability in *Saccharomyces cerevisiae*. 51, 657–668. https://doi.org/10.1080/10715762.2017.1355054

Gan, R.-Y., Chan, C.-L., Yang, Q.-Q., Li, H.-B., Zhang, D., Ge, Y.-Y., Gunaratne, A., Ge, J., Corke, H., 2019. 9 - Bioactive compounds and beneficial functions of sprouted grains, in: Feng, H., Nemzer, B., DeVries, J.W. (Eds.), Sprouted Grains. AACC International Press, pp. 191–246. https://doi.org/10.1016/B978-0-12-811525-1.00009-9

García-López, M.C., Mirón-García, M.C., Garrido-Godino, A.I., Mingorance, C., Navarro, F., 2010. Overexpression of SNG1 causes 6-azauracil resistance in *Saccharomyces cerevisiae*. Curr Genet 56, 251–263. https://doi.org/10.1007/s00294-010-0297-z

Gomes, A.M.V., Carmo, T.S., Carvalho, L.S., Bahia, F.M., Parachin, N.S., 2018. Comparison of Yeasts as Hosts for Recombinant Protein Production. Microorganisms 6. https://doi.org/10.3390/MICROORGANISMS6020038

Grey, M., Pich, C.T., Haase, E., Brendel, M., 1995. SNG1 — a new gene involved in nitrosoguanidine resistance in *Saccharomyces cerevisiae*. Mutation Research Letters 346, 207–214. https://doi.org/10.1016/0165-7992(95)90037-3

Gu, H., Zhang, J., Bao, J., 2015. High tolerance and physiological mechanism of *Zymomonas mobilis* to phenolic inhibitors in ethanol fermentation of corncob residue. Biotechnol Bioeng 112, 1770–1782. https://doi.org/10.1002/bit.25603

Gu, H., Zhu, Y., Peng, Y., Liang, X., Liu, X., Shao, L., Xu, Y., Xu, Z., Liu, R., Li, J., 2019. Physiological mechanism of improved tolerance of *Saccharomyces cerevisiae* to lignin-derived phenolic acids in lignocellulosic ethanol fermentation by short-term adaptation. Biotechnol Biofuels 12, 268. https://doi.org/10.1186/s13068-019-1610-9

Guil-Guerrero, J.L., Ramos, L., Moreno, C., Zúñiga-Paredes, J.C., Carlosama-Yepez, M., Ruales, P., 2016. Antimicrobial activity of plant-food by-products: A review focusing on the tropics. Livest Sci 189, 32–49. https://doi.org/10.1016/J.LIVSCI.2016.04.021

Guo, Z., Olsson, L., 2016. Physiological responses to acid stress by *Saccharomyces cerevisiae* when applying high initial cell density. FEMS Yeast Res 16, fow072. https://doi.org/10.1093/femsyr/fow072

He, Y., Zhang, J., Bao, J., 2016. Acceleration of biodetoxification on dilute acid pretreated lignocellulose feedstock by aeration and the consequent ethanol fermentation evaluation. Biotechnol Biofuels 9, 19. https://doi.org/10.1186/s13068-016-0438-9

Hill, P., Benjamin, K., Bhattacharjee, B., Garcia, F., Leng, J., Liu, C.L., Murarka, A., Pitera, D., Rodriguez Porcel, E.M., da Silva, I., Kraft, C., 2020. Clean manufacturing powered by biology: how Amyris has deployed technology and aims to do it better. J Ind Microbiol Biotechnol 47, 965–975. https://doi.org/10.1007/S10295-020-02314-3

Hur, S.J., Lee, S.Y., Kim, Y.-C., Choi, I., Kim, G.-B., 2014. Effect of fermentation on the antioxidant activity in plant-based foods. Food Chem 160, 346–356. https://doi.org/10.1016/j.foodchem.2014.03.112

Jacobs, A., Auburger, S., Bahrs, E., Brauer-Siebrecht, W., Christen, O., Götze, P., Koch, H.J., Mußhoff, O., Rücknagel, J., Märländer, B., 2017. Replacing silage maize for biogas production by sugar beet – A system analysis with ecological and economical approaches. Agric Syst 157, 270–278. https://doi.org/10.1016/J.AGSY.2016.10.004

Joannis-Cassan, C., Riess, J., Jolibert, F., Taillandier, P., 2014. Optimization of very high gravity fermentation process for ethanol production from industrial sugar beet syrup. Biomass Bioenergy 70, 165–173. https://doi.org/10.1016/J.BIOMBIOE.2014.07.027

Jönsson, L.J., Martín, C., 2016. Pretreatment of lignocellulose: Formation of inhibitory by-products and strategies for minimizing their effects. Bioresour Technol 199, 103–112. https://doi.org/10.1016/j.biortech.2015.10.009

Kalinowska, M., Gołębiewska, E., Świderski, G., Męczyńska-Wielgosz, S., Lewandowska, H., Pietryczuk, A., Cudowski, A., Astel, A., Świsłocka, R., Samsonowicz, M., Złowodzka, A.B., Priebe, W., Lewandowski, W., 2021. Plant-derived and dietary hydroxybenzoic acids—a comprehensive study of structural, anti-/pro-oxidant, lipophilic, antimicrobial, and cytotoxic activity in mda-mb-231 and mcf-7 cell lines. Nutrients 13, 3107. https://doi.org/10.3390/NU13093107/S1

Kim, J.H., Campbell, B.C., Mahoney, N., Chan, K.L., May, G.S., 2006. Targeting antioxidative signal transduction and stress response system: control of pathogenic Aspergillus with phenolics that inhibit mitochondrial function. J Appl Microbiol 101, 181–189. https://doi.org/10.1111/j.1365-2672.2006.02882.x

Kim, Y., Kreke, T., Hendrickson, R., Parenti, J., Ladisch, M.R., 2013. Fractionation of cellulase and fermentation inhibitors from steam pretreated mixed hardwood. Bioresour Technol 135, 30–38. https://doi.org/10.1016/j.biortech.2012.10.130

Krishnan, S., Homroskla, P., Saritpongteeraka, K., Suttinun, O., Nasrullah, M., Tirawanichakul, Y., Chaiprapat, S., 2023. Specific degradation of phenolic compounds from palm oil mill effluent using ozonation and its impact on methane fermentation. Chemical Engineering Journal 451, 138487. https://doi.org/10.1016/J.CEJ.2022.138487

Kumar, N., Goel, N., 2019. Phenolic acids: Natural versatile molecules with promising therapeutic applications. Biotechnology Reports 24, e00370. https://doi.org/10.1016/J.BTRE.2019.E00370

Kwak, S., Jin, Y.-S., 2017. Production of fuels and chemicals from xylose by engineered *Saccharomyces cerevisiae*: a review and perspective. Microb Cell Fact 16, 82. https://doi.org/10.1186/s12934-017-0694-9

Larsson, S., Nilvebrant, N.-O., Jönsson, L., 2001. Effect of overexpression of *Saccharomyces cerevisiae* Pad1p on the resistance to phenylacrylic acids and lignocellulose hydrolysates under aerobic and oxygen-limited conditions. Appl Microbiol Biotechnol 57, 167–174. https://doi.org/https://doi.org/10.1007/s002530100742

Leavell, M.D., McPhee, D.J., Paddon, C.J., 2016. Developing fermentative terpenoid production for commercial usage. Curr Opin Biotechnol 37, 114–119. https://doi.org/10.1016/J.COPBIO.2015.10.007

Lima, V.N., Oliveira-Tintino, C.D.M., Santos, E.S., Morais, L.P., Tintino, S.R., Freitas, T.S., Geraldo, Y.S., Pereira, R.L.S., Cruz, R.P., Menezes, I.R.A., Coutinho, H.D.M., 2016. Antimicrobial and enhancement of the antibiotic activity by phenolic compounds: Gallic acid, caffeic acid and pyrogallol. Microb Pathog 99, 56–61. https://doi.org/10.1016/J.MICPATH.2016.08.004

Lin, R., Cheng, J., Ding, L., Song, W., Zhou, J., Cen, K., 2015. Inhibitory effects of furan derivatives and phenolic compounds on dark hydrogen fermentation. Bioresour Technol 196, 250–255. https://doi.org/10.1016/j.biortech.2015.07.097

Li, Q., Bai, Z., O'Donnell, A., Harvey, L.M., Hoskisson, P.A., McNeil, B., 2011. Oxidative stress in fungal fermentation processes: The roles of alternative respiration. Biotechnol Lett 33, 457–467. https://doi.org/10.1007/S10529-010-0471-X/FIGURES/2

Liu, Y., Liu, G., Zhang, J., Balan, V., Bao, J., 2020. Itaconic acid fermentation using activated charcoal-treated corn stover hydrolysate and process evaluation based on Aspen plus model. Biomass Convers Biorefin 10, 463–470. https://doi.org/10.1007/S13399-019-00423-3/FIGURES/4

Liu, Y., Wang, Z., Cui, Z., Qi, Q., Hou, J., 2022. Progress and perspectives for microbial production of farnesene. Bioresour Technol 347, 126682. https://doi.org/10.1016/J.BIORTECH.2022.126682

Li, Y.-C., Gou, Z.-X., Zhang, Y., Xia, Z.-Y., Tang, Y.-Q., Kida, K., 2017. Inhibitor tolerance of a recombinant flocculating industrial *Saccharomyces cerevisiae* strain during glucose and xylose co-fermentation. Braz J Microbiol 48, 791–800. https://doi.org/10.1016/j.bjm.2016.11.011

López, P.C., Peng, C., Arneborg, N., Junicke, H., Gernaey, K. V., 2021. Analysis of the response of the cell membrane of *Saccharomyces cerevisiae* during the detoxification of common lignocellulosic inhibitors. Scientific Reports 2021 11:1 11, 1–16. https://doi.org/10.1038/s41598-021-86135-z

Maphetu, N., Unuofin, J.O., Masuku, N.P., Olisah, C., Lebelo, S.L., 2022. Medicinal uses, pharmacological activities, phytochemistry, and the molecular mechanisms of *Punica granatum* L. (pomegranate) plant extracts: A review. Biomedicine & Pharmacotherapy 153, 113256. https://doi.org/10.1016/J.BIOPHA.2022.113256

Maurício Duarte-Almeida, J., Novoa, A.V., Linares, A.F., Lajolo, F.M., Inés Genovese, M., 2006. Antioxidant Activity of Phenolics Compounds From Sugar Cane (*Saccharum officinarum* L.) Juice. Plant Foods for Human Nutrition 61, 187. https://doi.org/10.1007/s11130-006-0032-6

Meadows, A.L., Hawkins, K.M., Tsegaye, Y., Antipov, E., Kim, Y., Raetz, L., Dahl, R.H., Tai, A., Mahatdejkul-Meadows, T., Xu, L., Zhao, L., Dasika, M.S., Murarka, A., Lenihan, J., Eng, D., Leng, J.S., Liu, C.-L., Wenger, J.W., Jiang, H., Chao, L., Westfall, P., Lai, J., Ganesan, S., Jackson, P., Mans, R., Platt, D., Reeves, C.D., Saija, P.R., Wichmann, G., Holmes, V.F., Benjamin, K., Hill, P.W., Gardner, T.S., Tsong, A.E., 2016. Rewriting yeast central carbon metabolism for industrial isoprenoid production. Nature 537, 694–697. https://doi.org/10.1038/nature19769

Mehta, S., Joghi, S., Gowder, T., 2015. Members of Antioxidant Machinery and Their Functions. https://doi.org/10.5772/61884

Merkl, R., Hrádková, I., Filip, V., Šmidrkal, J., 2010. Antimicrobial and antioxidant properties of phenolic acids alkyl esters. Czech Journal of Food Sciences 28 (2010), 275–279. https://doi.org/10.17221/132/2010-CJFS

Mesquita, T.J.B., Sargo, C.R., Fuzer, J.R., Paredes, S.A.H., Giordano, R. de C., Horta, A.C.L., Zangirolami, T.C., 2019. Metabolic fluxes-oriented control of bioreactors: a novel approach to tune micro-aeration and substrate feeding in fermentations. Microb Cell Fact 18, 150. https://doi.org/10.1186/s12934-019-1198-6

Murkovic, M., 2003. PHENOLIC COMPOUNDS, in: Caballero, B. (Ed.), Encyclopedia of Food Sciences and Nutrition (Second Edition). Academic Press, Oxford, pp. 4507–4514. https://doi.org/10.1016/B0-12-227055-X/00914-7

Niesen, D.B., Hessler, C., Seeram, N.P., 2013. Beyond resveratrol: A review of natural stilbenoids identified from 2009–2013. J Berry Res 3, 181–196. https://doi.org/10.3233/JBR-130062

O'Donnell, A., Bai, Y., Bai, Z., McNeil, B., Harvey, L.M., 2007. Introduction to bioreactors of shake-flask inocula leads to development of oxidative stress in *Aspergillus niger*. Biotechnol Lett 29, 895–900. https://doi.org/10.1007/S10529-007-9336-3/FIGURES/3

Oliveira, A., Gomes, M.H., Alexandre, E.M.C., Poças, F., Almeida, D.P.F., Pintado, M., 2015. Phytochemicals preservation in strawberry as affected by pH modulation. Food Chem 170, 74–83. https://doi.org/10.1016/j.foodchem.2014.07.156

Ortega, J.O., Mora Vargas, J.A., Metzker, G., Gomes, E., da Silva, R., Boscolo, M., 2021. Enhancing the production of the fermentable sugars from sugarcane straw: A new approach to applying alkaline and ozonolysis pretreatments. Renew Energy 164, 502–508. https://doi.org/10.1016/j.renene.2020.09.070

Pan, L., He, M., Wu, B., Wang, Y., Hu, G., Ma, K., 2019. Simultaneous concentration and detoxification of lignocellulosic hydrolysates by novel membrane filtration system for bioethanol production. J Clean Prod 227, 1185–1194. https://doi.org/10.1016/J.JCLEPRO.2019.04.239

Parapouli, M., Vasileiadis, A., Afendra, A.S., Hatziloukas, E., 2020. *Saccharomyces cerevisiae* and its industrial applications. AIMS Microbiol 6, 1. https://doi.org/10.3934/MICROBIOL.2020001

Park, E.-H., Kim, M.-D., 2014. Genome-Wide Screening of *Saccharomyces cerevisiae* Genes Regulated by Vanillin. J Microbiol Biotechnol 25. https://doi.org/10.4014/jmb.1409.09064

Pizzolitto, R.P., Barberis, C.L., Dambolena, J.S., Herrera, J.M., Zunino, M.P., Magnoli, C.E., Rubinstein, H.R., Zygadlo, J.A., Dalcero, A.M., 2015. Inhibitory Effect of Natural Phenolic Compounds on *Aspergillus parasiticus* Growth. J Chem 2015, 547925. https://doi.org/10.1155/2015/547925

Rahmanian, N., Jafari, S.M., Galanakis, C.M., 2014. Recovery and removal of phenolic compounds from olive mill wastewater. JAOCS, Journal of the American Oil Chemists' Society 91, 1–18. https://doi.org/10.1007/S11746-013-2350-9/TABLES/8

Rawoof, S.A.A., Kumar, P.S., Vo, D.V.N., Devaraj, K., Mani, Y., Devaraj, T., Subramanian, S., 2020. Production of optically pure lactic acid by microbial fermentation: a review. Environmental Chemistry Letters 2020 19:1 19, 539–556. https://doi.org/10.1007/S10311-020-01083-W

Raza, W., Lee, J., Raza, N., Luo, Y., Kim, K.H., Yang, J., 2019. Removal of phenolic compounds from industrial waste water based on membrane-based technologies. Journal of Industrial and Engineering Chemistry 71, 1–18. https://doi.org/10.1016/J.JIEC.2018.11.024

Renouf, M.A., Wegener, M.K., Nielsen, L.K., 2008. An environmental life cycle assessment comparing Australian sugarcane with US corn and UK sugar beet as producers of sugars for fermentation. Biomass Bioenergy 32, 1144–1155. https://doi.org/10.1016/J.BIOMBIOE.2008.02.012

Richard, P., Viljanen, K., Penttilä, M., 2015. Overexpression of PAD1 and FDC1 results in significant cinnamic acid decarboxylase activity in *Saccharomyces cerevisiae*. AMB Express 5, 12. https://doi.org/10.1186/s13568-015-0103-x

Sadowska-Bartosz, I., Bartosz, G., 2014. Effect of antioxidants supplementation on aging and longevity. Biomed Res Int 2014, 404680. https://doi.org/10.1155/2014/404680

Šamec, D., Karalija, E., Šola, I., Vujčić Bok, V., Salopek-Sondi, B., 2021. The Role of Polyphenols in Abiotic Stress Response: The Influence of Molecular Structure. Plants 2021, Vol. 10, Page 118 10, 118. https://doi.org/10.3390/PLANTS10010118

Sánchez, Ó.J., Cardona, C.A., 2008. Trends in biotechnological production of fuel ethanol from different feedstocks. Bioresour Technol 99, 5270–5295. https://doi.org/10.1016/J.BIORTECH.2007.11.013

Singh, A., Lal, U.R., Mukhtar, H.M., Singh, P.S., Shah, G., Dhawan, R.K., 2015. Phytochemical profile of sugarcane and its potential health aspects. Pharmacogn Rev 9, 45–54. https://doi.org/10.4103/0973-7847.156340

Soares, L.B., Bonan, C.I.D.G., Biazi, L.E., Dionísio, S.R., Bonatelli, M.L., Andrade, A.L.D., Renzano, E.C., Costa, A.C., Ienczak, J.L., 2020. Investigation of hemicellulosic hydrolysate inhibitor resistance and fermentation strategies to overcome inhibition in non-saccharomyces species. Biomass Bioenergy 137, 105549. https://doi.org/10.1016/j.biombioe.2020.105549

Sotler, R., Poljšak, B., Dahmane, R., Jukić, T., Pavan Jukić, D., Rotim, C., Trebše, P., Starc, A., 2019. PROOXIDANT ACTIVITIES OF ANTIOXIDANTS AND THEIR IMPACT ON HEALTH. Acta Clin Croat 58, 726. https://doi.org/10.20471/ACC.2019.58.04.20

Terpinc, P., Abramovič, H., 2010. A kinetic approach for evaluation of the antioxidant activity of selected phenolic acids. Food Chem 121, 366–371. https://doi.org/10.1016/J.FOODCHEM.2009.12.037

Thevissen, K., Terras, F.R.G., Broekaert, W.F., 1999. Permeabilization of fungal membranes by plant defensins inhibits fungal growth. Appl Environ Microbiol 65, 5451–5458. https://doi.org/10.1128/AEM.65.12.5451-5458.1999/

Vuolo, M.M., Lima, V.S., Maróstica Junior, M.R., 2019. Chapter 2 - Phenolic Compounds: Structure, Classification, and Antioxidant Power, in: Campos, M.R.S. (Ed.), Bioactive Compounds. Woodhead Publishing, pp. 33–50. https://doi.org/10.1016/B978-0-12-814774-0.00002-5

Wang, M., Wang, Zhaoqing, Li, S., Wang, Zhong, Zhao, J., 2015. Mediated electrochemical method for the analysis of membrane damage effects of phenolic compounds to

Staphylococcus aureus. Journal of Electroanalytical Chemistry 757, 44–50. https://doi.org/10.1016/j.jelechem.2015.09.007

Wang, X., Liang, Z., Hou, J., Bao, X., Shen, Y., 2016. Identification and functional evaluation of the reductases and dehydrogenases from *Saccharomyces cerevisiae* involved in vanillin resistance. BMC Biotechnol 16, 31. https://doi.org/10.1186/s12896-016-0264-y

Wang, X., Tsang, Y.F., Li, Y., Ma, X., Cui, S., Zhang, T.-A., Hu, J., Gao, M.-T., 2017. Inhibitory effects of phenolic compounds of rice straw formed by saccharification during ethanol fermentation by *Pichia stipitis*. Bioresour Technol 244, 1059–1067. https://doi.org/10.1016/j.biortech.2017.08.096

Wohler Sunnarborg, S., Miller, S.P., Unnikrishnan, I., LaPorte, D.C., 2001. Expression of the yeast glycogen phosphorylase gene is regulated by stress-response elements and by the HOG MAP kinase pathway. Yeast 18, 1505–1514. https://doi.org/10.1002/yea.752

Wu, N., Xing, M., Chen, Y., Zhang, C., Li, Y., Song, P., Xu, Q., Liu, H., Huang, H., 2022. Improving the productivity of malic acid by alleviating oxidative stress during *Aspergillus niger* fermentation. Biotechnology for Biofuels and Bioproducts 15, 1–16. https://doi.org/10.1186/S13068-022-02250-7

Xie, R., Tu, M., Elder, T., 2016. Substituent Effect of Phenolic Aldehyde Inhibition on Alcoholic Fermentation by *Saccharomyces cerevisiae*. Energy & Fuels 30, 3078–3084. https://doi.org/10.1021/acs.energyfuels.5b03034

Yankov, D., 2022. Fermentative Lactic Acid Production From Lignocellulosic Feedstocks: From Source to Purified Product. Front Chem 10, 823005. https://doi.org/10.3389/FCHEM.2022.823005

Yu, X.D., Pickett, J., Ma, Y.Z., Bruce, T., Napier, J., Jones, H.D., Xia, L.Q., 2012. Metabolic Engineering of Plant-derived (E)-β-farnesene Synthase Genes for a Novel Type of Aphid-resistant Genetically Modified Crop PlantsF. J Integr Plant Biol 54, 282–299. https://doi.org/10.1111/J.1744-7909.2012.01107.X

Zabed, H., Faruq, G., Sahu, J.N., Azirun, M.S., Hashim, R., Nasrulhaq Boyce, A., 2014. Bioethanol Production from Fermentable Sugar Juice. The Scientific World Journal 2014, 957102. https://doi.org/10.1155/2014/957102

Zhang, L., Li, X., Yong, Q., Yang, S.-T., Ouyang, J., Yu, S., 2016. Impacts of lignocellulose-derived inhibitors on l-lactic acid fermentation by *Rhizopus oryzae*. Bioresour Technol 203, 173–180. https://doi.org/10.1016/j.biortech.2015.12.014

Chapter 2

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

Luís Carlos Carvalho[a,b], Ana L. S. Oliveira[b], Erdem Carsanba[a,b], Manuela Pintado[b], Carla Oliveira[b*]

[a] Amyris BioProducts Portugal, Unipessoal, Lda. Rua Diogo Botelho, 1327, 4169-005 Porto, Portugal

[b] CBQF - Centro de Biotecnologia e Química Fina - Laboratório Associado, Escola Superior de Biotecnologia, Universidade Católica Portuguesa, Rua Diogo Botelho, 1327, 4169-005 Porto, Portugal

Published

Industrial Crops and Products

2022, Volume 188, Part B

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 a 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 strongly inhibit 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 by 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 an 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 feed rate 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.

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

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.

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

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 3). 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.

Table 3. 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.

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

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 4). 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

Table 4. 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.

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

the other hand, all phenolic compounds have also been identified as antioxidant agents, such as feruloylquinic acids, with an ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) 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).

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 4). 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 4) 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 7A), 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 7A) 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 7B), 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).

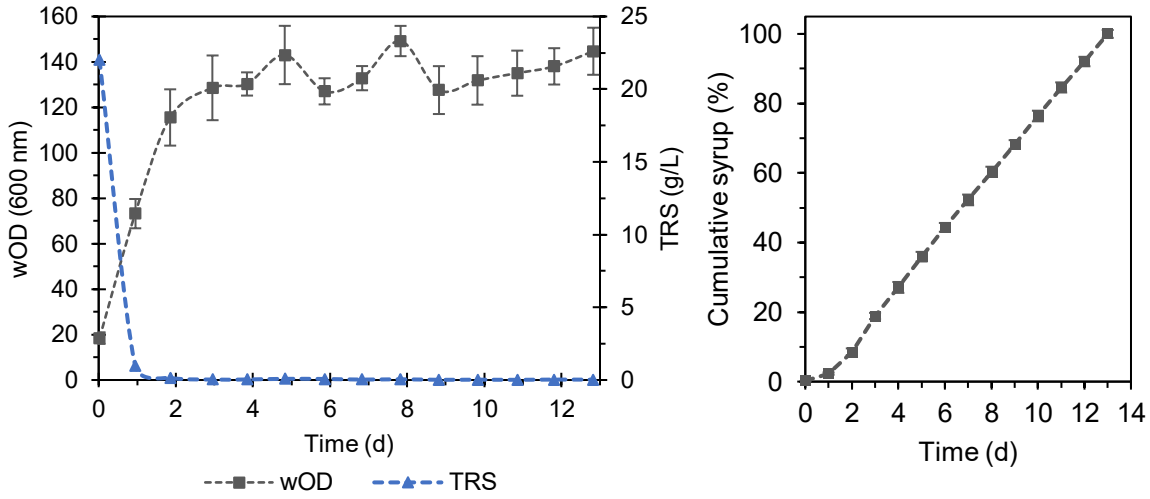

Figure 7. Washed optical density measured at 600 nm and total reducing sugars (TRS) (A), and normalized cumulative sugarcane syrup (B) through 13 days of fed-batch fermentations of farnesene producing *S. cerevisiae*. Results represent the average of 4 bioreactor fermentations and error bars are standard 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 8). The mass of syrup added into the bioreactors increased over time (Figure 7B). 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 7A). 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.

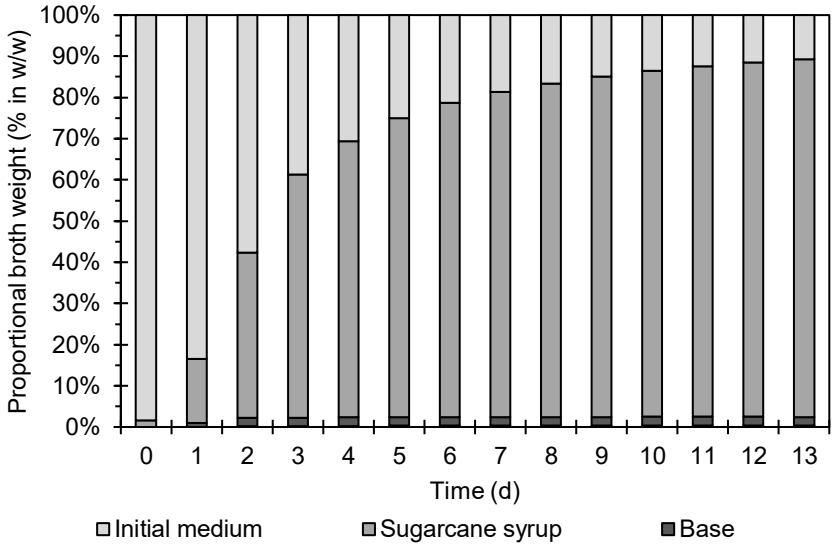

Figure 8. Proportional broth weight (%) of all medium components (initial medium, sugarcane syrup and base) during 13 days of fed-batch fermentations of farnesene producing *S. cerevisiae*. Results represent the average of 4 bioreactor fermentations and error bars are standard deviations.

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 (gfarnesene/gsugar) 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 9). 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 9). 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.

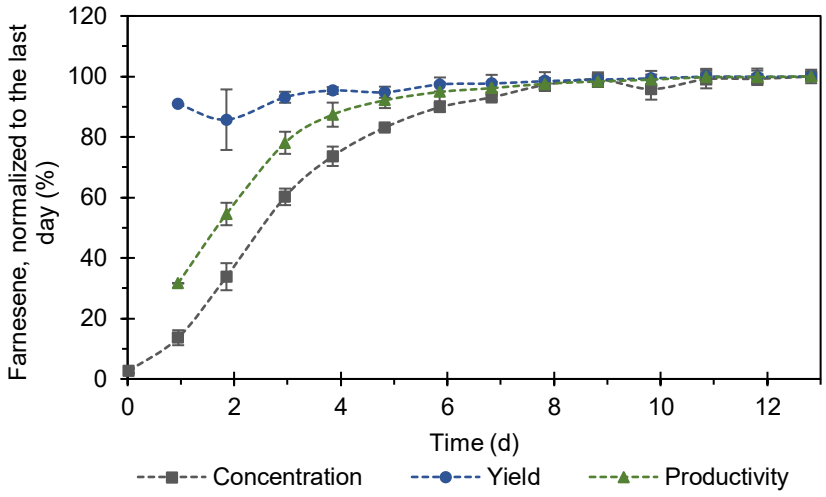

Figure 9. Farnesene concentration (g farnesene/L broth), yield (g farnesene/ g TRS) and productivity (g farnesene/L broth/ h) normalized in relation to the last day of fermentation during 13 days of fed-batch fermentations of farnesene producing *S. cerevisiae*. Results represent the average of 4 bioreactor 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 10. 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.

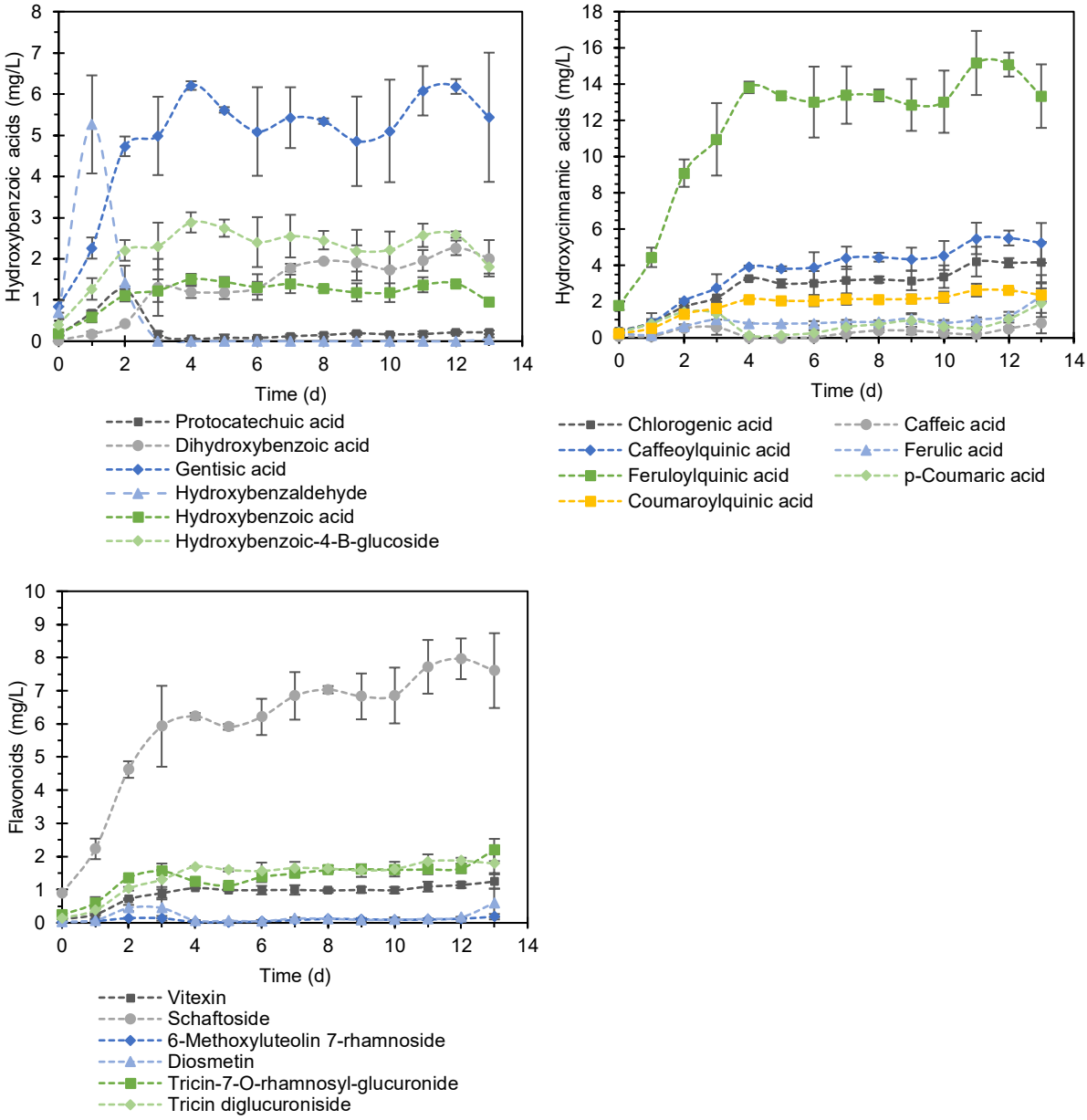

Figure 10. Identified phenolic compounds measured in LC-ESI-QqTOF-HRMS during 13 days of fed-batch fermentations of farnesene producing *S. cerevisiae*. A – hydroxybenzoic acids, B – hydroxycinnamic acids, C – flavonoids. Results represent the average of 4 bioreactor fermentations and error bars are 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 11 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 3). This means that, in the β-farnesene fermentation process, phenolic compounds are accumulating to the levels found in the feedstock.

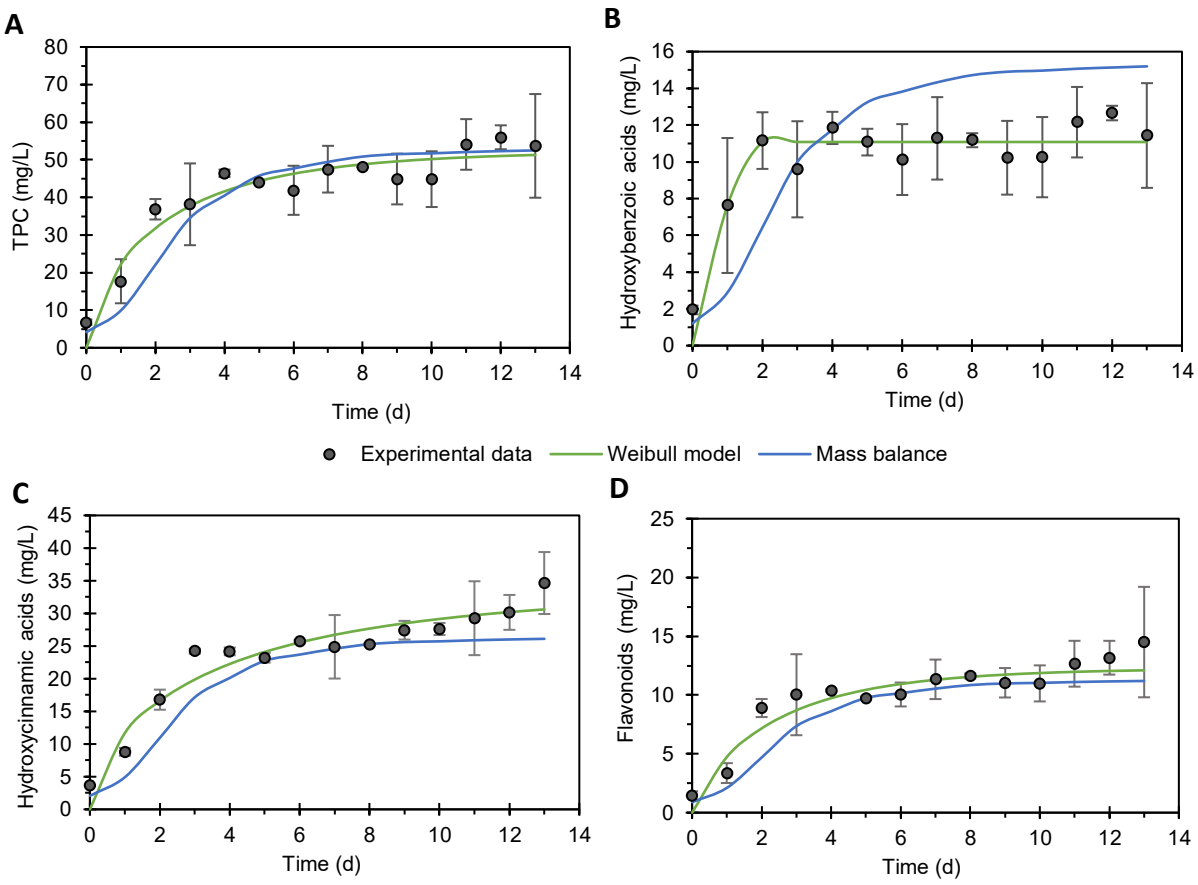

Figure 11. Total phenolic content (TPC) (A), hydroxybenzoic acids (B), hydroxycinnamic acids (C) and flavonoids (D) measured in LC-ESI-UHR-QqTOF-MS during 13 days of fed-batch fermentations of farnesene producing *S. cerevisiae*. Plotted dots represent the data for the average of 4 reactor fermentations, 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

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 5). This model presented a good adjustment to the experimental data during the fermentation time (Figure 11). 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).

Table 5. Parameters for Weibull model fitting to phenolic compound experimental data. Estimates (± standard deviation) of Weibull model parameters used to describe total phenolic content (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 TPC and phenolic classes (A) and each individual phenolic compound (a).

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

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 the syrup (Table 6). No significant differences have been found between both batches of syrup. The fit of this calculation presented a similar profile to the experimental data, with an increase of phenolic compounds over the fermentation time (Figure 11). Regarding the fit to data of TPC, hydroxycinnamic acids and flavonoids, it presented an R^2 of 0.86 or higher. However, the poorest adjustment to the experimental data was obtained for the hydroxybenzoic acids class, with an R^2 of 0.63. This result can be explained by the fact that the mass balance does not consider any degradation of the molecules, which was clear in this phenolic class. The decrease of the concentration of certain phenolic compounds during the fermentation revealed that the evolution of phenolic compounds in the studied fed-batch fermentation does not just follow a mass balance profile, with the concentration being augmented as syrup is added to the bioreactor, but that the conversion of molecules by the yeast must be also considered.

Table 6. Coefficient of determination and root mean square error for theoretical mass balance fitting to phenolic compounds (total phenolic content (TPC), total hydroxybenzoic acids, hydroxycinnamic acids and flavonoids) during the fed-batch fermentation using sugarcane syrup as feedstock.

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

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

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

4. Conclusions

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

References

Abdel-Aleem, W. 2020. Effect of Sugarcane Juice Pre-treatment on the Quality and Crystallization of Sugarcane Syrup. J Food Process Technol, 11, 834.

Adeboye, P.T., Bettiga, M., Olsson, L. 2014. The chemical nature of phenolic compounds determines their toxicity and induces distinct physiological responses in Saccharomyces cerevisiae in lignocellulose hydrolysates. AMB Express, 4(1), 46.

Adeboye, P.T., Bettiga, M., Aldaeus, F., Larsson, P.T., Olsson, L. 2015. Catabolism of coniferyl aldehyde, ferulic acid and p-coumaric acid by Saccharomyces cerevisiae yields less toxic products. Microbial cell factories, 14, 149-149.

Agu, K., Oduola, K. 2021. Kinetic modeling of ethanol production by batch fermentation of sugarcane juice using immobilized yeast. Global Journal of Engineering and Technology Advances, 7, 124-136.

Alonso-Riaño, P., Sanz Diez, M.T., Blanco, B., Beltrán, S., Trigueros, E., Benito-Román, O. 2020. Water Ultrasound-Assisted Extraction of Polyphenol Compounds from Brewer's Spent Grain: Kinetic Study, Extract Characterization, and Concentration. Antioxidants, 9(3).

Bajko, E., Kalinowska, M., Borowski, P., Siergiejczyk, L., Lewandowski, W. 2015. 5-O-Caffeoylquinic acid: A spectroscopic study and biological screening for antimicrobial activity. LWT - Food Science and Technology, 65.

Baptista, S.L., Carvalho, L.C., Romaní, A., Domingues, L. 2020. Development of a sustainable bioprocess based on green technologies for xylitol production from corn cob. Industrial Crops and Products, 156, 112867.

Barrera, C., Betoret, N., Seguí, L. 2020. Phenolic Profile of Cane Sugar Derivatives Exhibiting Antioxidant and Antibacterial Properties. Sugar Tech, 22.

Carsanba, E., Pintado, M., Oliveira, C. 2021. Fermentation Strategies for Production of Pharmaceutical Terpenoids in Engineered Yeast. Pharmaceuticals, 14(4).

Coutinho, I.D., Baker, J.M., Ward, J.L., Beale, M.H., Creste, S., Cavalheiro, A.J. 2016. Metabolite Profiling of Sugarcane Genotypes and Identification of Flavonoid Glycosides and Phenolic Acids. Journal of Agricultural and Food Chemistry, 64(21), 4198-4206.

Deseo, M.A., Elkins, A., Rochfort, S., Kitchen, B. 2020. Antioxidant activity and polyphenol composition of sugarcane molasses extract. Food Chem, 314, 126180.

Duarte-Almeida, J.M., Novoa, A.V., Linares, A.F., Lajolo, F.M., Inés Genovese, M. 2006. Antioxidant Activity of Phenolics Compounds From Sugar Cane (Saccharum officinarum L.) Juice. Plant Foods for Human Nutrition, 61(4), 187.

Duarte-Almeida, J.M., Salatino, A., Genovese, M.I., Lajolo, F.M. 2011. Phenolic composition and antioxidant activity of culms and sugarcane (Saccharum officinarum L.) products. Food Chemistry, 125(2), 660-664.

Eggleston, G. 2018. Positive Aspects of Cane Sugar and Sugar Cane Derived Products in Food and Nutrition. J Agric Food Chem, 66(16), 4007-4012.

Farhoosh, R., Johnny, S., Asnaashari, M., Molaahmadibahraseman, N., Sharif, A. 2016. Structure–antioxidant activity relationships of o-hydroxyl, o-methoxy, and alkyl ester derivatives of p-hydroxybenzoic acid. Food Chemistry, 194, 128-134.

Fletcher, E., Gao, K., Mercurio, K., Ali, M., Baetz, K. 2019. Yeast chemogenomic screen identifies distinct metabolic pathways required to tolerate exposure to phenolic fermentation inhibitors ferulic acid, 4-hydroxybenzoic acid and coniferyl aldehyde. Metabolic Engineering, 52, 98-109.

Germec, M., Karhan, M., Bialka, K.L., Demirci, A., Turhan, I. 2018. Mathematical modeling of lactic acid fermentation in bioreactor with carob extract. Biocatalysis and Agricultural Biotechnology, 14, 254-263.

Gu, H., Zhang, J., Bao, J. 2015. High tolerance and physiological mechanism of Zymomonas mobilis to phenolic inhibitors in ethanol fermentation of corncob residue. Biotechnology and Bioengineering, 112(9), 1770-1782.

Hammoda, H.M., Ghazy, N.M., Harraz, F.M., Radwan, M.M., ElSohly, M.A., Abdallah, I.I. 2013. Chemical constituents from Tribulus terrestris and screening of their antioxidant activity. Phytochemistry, 92, 153-159.

Huang, X.-x., Zhu, G.-q., Liu, Q., Chen, L., Li, Y.-j., Hou, B.-k. 2018. Modulation of Plant Salicylic Acid-Associated Immune Responses via Glycosylation of Dihydroxybenzoic Acids1. Plant Physiology, 176(4), 3103-3119.

Hur, S.J., Lee, S.Y., Kim, Y.-C., Choi, I., Kim, G.-B. 2014. Effect of fermentation on the antioxidant activity in plant-based foods. Food Chemistry, 160, 346-356.

Jönsson, L.J., Palmqvist, E., Nilvebrant, N.O., Hahn-Hägerdal, B. 1998. Detoxification of wood hydrolysates with laccase and peroxidase from the white-rot fungus Trametes versicolor. Applied Microbiology and Biotechnology, 49(6), 691-697.

Karamać, M., Kosińska, A., Estrella, I., Hernández, M.T., Dueñas, M. 2012. Antioxidant activity of phenolic compounds identified in sunflower seeds.

Khole, S., A Panat, N., Suryawanshi, P., Chatterjee, S., Devasagayam, T.P.A., Ghaskadbi, S. 2016. Comprehensive Assessment of Antioxidant Activities of Apigenin Isomers: Vitexin and Isovitexin. Free Radicals and Antioxidants, 6(2), 155-166.

Kim, Y., Kreke, T., Hendrickson, R., Parenti, J., Ladisch, M.R. 2013. Fractionation of cellulase and fermentation inhibitors from steam pretreated mixed hardwood. Bioresource Technology, 135, 30-38.

McNally, D.J., Wurms, K.V., Labbé, C., Quideau, S., Bélanger, R.R. 2003. Complex C-glycosyl flavonoid phytoalexins from Cucumis sativus. J Nat Prod, 66(9), 1280-3.

Meadows, A.L., Hawkins, K.M., Tsegaye, Y., Antipov, E., Kim, Y., Raetz, L., Dahl, R.H., Tai, A., Mahatdejkul-Meadows, T., Xu, L., Zhao, L., Dasika, M.S., Murarka, A., Lenihan, J., Eng, D., Leng, J.S., Liu, C.-L., Wenger, J.W., Jiang, H., Chao, L., Westfall, P., Lai, J., Ganesan, S., Jackson, P., Mans, R., Platt, D., Reeves, C.D., Saija, P.R., Wichmann, G., Holmes, V.F., Benjamin, K., Hill, P.W., Gardner, T.S., Tsong, A.E. 2016. Rewriting yeast central carbon metabolism for industrial isoprenoid production. Nature, 537(7622), 694-697.

Merkl, R., Hradkova, I., Filip, V., Šmidrkal, J. 2010. Antimicrobial and Antioxidant Properties of Phenolic Acids Alkyl Esters. Czech Journal of Food Sciences, 28, 275-279.

Mesquita, T.J.B., Sargo, C.R., Fuzer, J.R., Paredes, S.A.H., Giordano, R.d.C., Horta, A.C.L., Zangirolami, T.C. 2019. Metabolic fluxes-oriented control of bioreactors: a novel approach to tune micro-aeration and substrate feeding in fermentations. Microbial Cell Factories, 18(1), 150.

Murkovic, M. 2003. PHENOLIC COMPOUNDS. in: Encyclopedia of Food Sciences and Nutrition (Second Edition), (Ed.) B. Caballero, Academic Press. Oxford, pp. 4507-4514.

Nobsathian, S., Tuchinda, P., Sobhon, P., Tinikul, Y., Poljaroen, J., Tinikul, R., Sroyraya, M., Poomton, T., Chaichotranunt, S. 2017. An antioxidant activity of the whole body of Holothuria scabra. Chemical and Biological Technologies in Agriculture, 4(1), 4.

Oliveira, A., Gomes, M.H., Alexandre, E.M.C., Poças, F., Almeida, D.P.F., Pintado, M. 2015. Phytochemicals preservation in strawberry as affected by pH modulation. Food Chemistry, 170, 74-83.

Paddon, C.J., Westfall, P.J., Pitera, D.J., Benjamin, K., Fisher, K., McPhee, D., Leavell, M.D., Tai, A., Main, A., Eng, D., Polichuk, D.R., Teoh, K.H., Reed, D.W., Treynor, T., Lenihan, J., Jiang, H., Fleck, M., Bajad, S., Dang, G., Dengrove, D., Diola, D., Dorin, G., Ellens, K.W., Fickes, S., Galazzo, J., Gaucher, S.P., Geistlinger, T., Henry, R., Hepp, M., Horning, T., Iqbal, T., Kizer, L., Lieu, B., Melis, D., Moss, N., Regentin, R., Secrest, S., Tsuruta, H., Vazquez, R., Westblade, L.F., Xu, L., Yu, M., Zhang, Y., Zhao, L., Lievense, J., Covello, P.S., Keasling, J.D., Reiling, K.K., Renninger, N.S., Newman, J.D. 2013. High-level semi-synthetic production of the potent antimalarial artemisinin. Nature, 496(7446), 528-532.

Payet, B., Shum Cheong Sing, A., Smadja, J. 2006. Comparison of the Concentrations of Phenolic Constituents in Cane Sugar Manufacturing Products with Their Antioxidant Activities. Journal of Agricultural and Food Chemistry, 54(19), 7270-7276.

Qureshi, N., Saha, B.C., Cotta, M.A. 2008. Butanol production from wheat straw by simultaneous saccharification and fermentation using Clostridium beijerinckii: Part II—Fed-batch fermentation. Biomass and Bioenergy, 32(2), 176-183.

Richard, P., Viljanen, K., Penttilä, M. 2015. Overexpression of PAD1 and FDC1 results in significant cinnamic acid decarboxylase activity in Saccharomyces cerevisiae. AMB Express, 5(1), 12.

Sadowska-Bartosz, I., Bartosz, G. 2014. Effect of antioxidants supplementation on aging and longevity. BioMed research international, 2014, 404680-404680.

Shang, Y.-F., Hyun Cha, K., Ha Lee, E., Pan, C.-H., Um, B.-H. 2015. Optimization, Bio accessibility of Tricin and Anti-oxidative Activity of Extract from Black Bamboo Leaves. Free Radicals and Antioxidants, 6(1), 64-71.

Subramaniam, R., Thirumal, V., Chistoserdov, A., Bajpai, R., Bader, J., Popovic, M. 2018. High-density Cultivation in the Production of Microbial Products. Chemical and Biochemical Engineering, 32(4), 451-464.

Tsuchiya, H. 2015. Membrane Interactions of Phytochemicals as Their Molecular Mechanism Applicable to the Discovery of Drug Leads from Plants. Molecules, 20(10).

van Boekel, M.A. 2002. On the use of the Weibull model to describe thermal inactivation of microbial vegetative cells. Int J Food Microbiol, 74(1-2), 139-59.

van Hoek, P., de Hulster E Fau - van Dijken, J.P., van Dijken Jp Fau - Pronk, J.T., Pronk, J.T. 2000. Fermentative capacity in high-cell-density fed-batch cultures of baker's yeast. (0006-3592 (Print)).

Vila, F.C., Colombo, R., de Lira, T.O., Yariwake, J.H. 2008. HPLC microfractionation of flavones and antioxidant (radical scavenging) activity of Saccharum officinarum L. Journal of the Brazilian Chemical Society, 19(5), 903-908.

Wang, H., Yang, L., Zu, Y., Zhao, X. 2014. Microwave-Assisted Simultaneous Extraction of Luteolin and Apigenin from Tree Peony Pod and Evaluation of Its Antioxidant Activity. The Scientific World Journal, 2014, 506971.

Weibull, W. 1951. A Statistical Distribution Function of Wide Applicability. ASME Journal of Applied Mechanics, 293-197.

Yang, Y.-J., Liu, X., Wu, H.-R., He, X.-F., Bi, Y.-R., Zhu, Y., Liu, Z.-L. 2013. Radical scavenging activity and cytotoxicity of active quinic acid derivatives from Scorzonera divaricata roots. Food Chemistry, 138(2), 2057-2063.

Zabed, H., Faruq, G., Sahu, J.N., Azirun, M.S., Hashim, R., Nasrulhaq Boyce, A. 2014. Bioethanol Production from Fermentable Sugar Juice. The Scientific World Journal, 2014, 957102.

Zhang, L., Li, X., Yong, Q., Yang, S.-T., Ouyang, J., Yu, S. 2016. Impacts of lignocellulose-derived inhibitors on l-lactic acid fermentation by Rhizopus oryzae. Bioresource Technology, 203, 173-180.

Zhou, J.-M., Ibrahim, R.K. 2010. Tricin—a potential multifunctional nutraceutical. Phytochemistry Reviews, 9(3), 413-424.

Chapter 3

Removal of phenolic compounds from sugarcane syrup and impact on *Saccharomyces cerevisiae* fermentation

Luís Carlos Carvalho[a,b], Ana L. S. Oliveira[b], Erdem Carsanba[a,b], Ana Lopes[a,b], Tânia Leal[b], Mónica Ribeiro[a,b], Sara Fernandes[b], Manuela Pintado[b], Carla Oliveira[b]

[a] Amyris BioProducts Portugal, Unipessoal, Lda. Rua Diogo Botelho, 1327, 4169-005 Porto, Portugal

[b] CBQF - Centro de Biotecnologia e Química Fina - Laboratório Associado, Escola Superior de Biotecnologia, Universidade Católica Portuguesa, Rua Diogo Botelho, 1327, 4169-005 Porto, Portugal

Submitted

Abstract

This work aimed to study for the first time the effects of phenolic compounds from sugarcane syrup on *Saccharomyces cerevisiae* β-farnesene fermentation by removing them from this feedstock. Syrup purification was optimized through a central composite design using 5 types of activated charcoal, 3 contact times (1 to 24 h) and 3 adsorbent concentrations (10 to 150 g/L). The optimal purification condition - charcoal pellets at 115 g/L and contact time of 12.5 h - led to 96.7 % of phenolic compounds removal and 43.7 % of syrup recovery. The effects of reducing phenolic content from approximately 7.0 to 0.3 mg/L in sugarcane syrup on yeast fermentation varied with the scale. Increase in biomolecule productivity was only observed in shake-flasks (11 %) and in biomass productivity only in 2 L bioreactor (12 %). Thus, phenolic compounds from sugarcane syrup do not influence β-farnesene production at large-scale, under the conditions tested.

Keywords: Phenolic compounds; Sugarcane syrup purification; Central composite design; *Saccharomyces cerevisiae*; β-Farnesene fermentation.

1. Introduction

Currently, plant syrups are widely used in industrial fermentations to biosynthesize value-added molecules. For instance, syrups from corn, sugarcane, sorghum and sugarbeet are supplied into industrial bioreactors to be used as feedstock in the production of valuable products, such as bioethanol and β-farnesene (Hill et al., 2020; Joannis-Cassan et al., 2014; Nghiem et al., 2016; Vohra et al., 2014). The use of these carbon sources makes the production process cheaper than using refined sucrose due to the lower cost of the purification process. However, since these materials contain other molecules in their composition rather than sugars, such as phenolic compounds, they are also transferred into the culture broth (Duarte-Almeida et al., 2011).

Phenolic compounds - also referred to as phenols – comprise a large group of molecules produced by plants as secondary metabolites, aiding in the protection of diseases, such as microbial infection (Huang et al., 2018). One consequence of the chemical nature of phenolic compounds is their antimicrobial activity, preventing the growth and metabolism of microorganisms (Adeboye et al., 2014). Studies have shown that smaller molecular weight phenolic compounds, including monophenols, have a stronger antimicrobial activity than higher molecular weight polyphenols (Almeida et al., 2007; Kim et al., 2013; Lin et al., 2015; Wang et al., 2017). Moreover, it has been discovered that lower polarity boosts antimicrobial activity of these compounds. For instance, *Saccharomyces cerevisiae* is more inhibited by butyl esters of phenolic acids than methyl esters (Guil-Guerrero et al., 2016; Merkl et al., 2010).

On the other hand, phenolic compounds present in the culture medium prevent the accumulation of free radicals and reactive oxygen species (ROS) inside the microbial cells (Duarte-Almeida et al., 2011). Oxidative stress, which is a consequence of the generation of these radicals, has been correlated with cell aging. The prevention of ROS accumulation through antioxidant activity of these compounds can avoid cell apoptosis (Juan et al., 2021). However, this effect depends on which molecules are present. For instance, polyphenolic compounds, which contain multiple hydroxyl groups in their structure, are more effective antioxidants than monophenols. An example of polyphenolic compounds with excellent antioxidant activity is the class of flavonoids (Hur et al., 2014).

The phenolic compounds present in the fermentation broth may influence the process in two ways: lowering the fermentation performance due to their antimicrobial activity or improving it due to their antioxidant activity (Adeboye et al., 2014; Duarte-Almeida et al., 2011). Consequently, industrial fermentations that use syrups containing phenolic compounds may be impacted by their presence. For instance, sugarcane syrup, used by *S. cerevisiae* to produce β-farnesene, was reported by our group to contain a total phenolic content (TPC) of around 50 mg/L (Carvalho et al., 2022). The same concentration was also present in the culture broth at the last stage of 13-days fed-batch fermentation (Carvalho et al., 2022). In this study, dozens of individual phenolic compounds were identified in the syrup which were divided into three classes: hydroxybenzoic acids (HBA; 14.20 mg/L), hydroxycinnamic acids (HCA; 28.22 mg/L) and flavonoids (12.35 mg/L). However, the impact of this mixture of phenolic compounds on yeast fermentation is still unknown. It would be important to understand if these molecules affect the fermentation process, and one way to elucidate this is to remove the phenolic compounds from the sugarcane syrup and compare the fermentation performance between the two conditions, i.e., fermentation with purified syrup versus fermentation with non-purified syrup.

Several methods have been described in the literature to remove phenolic compounds from aqueous solutions, such as over-liming, evaporation, ion exchange resins and adsorption by activated charcoal (Canilha et al., 2010; Mushtaq et al., 2019). Among these methods, activated charcoal has shown a high adsorption capacity, while being inexpensive and easy to apply in the removal of the desired compounds (Kamal et al., 2011). For instance, adsorption through activated charcoal has been applied to purify a by-product from the sugarcane industry, namely sugarcane bagasse hydrolysate (Deng et al., 2018). The charcoal used in that study slightly reduced the content of sugar while removing inhibitory chemicals like phenolic compounds. However, to the best knowledge of the authors, the removal of phenolic compounds from sugarcane syrup has not yet been studied.

Therefore, this work aimed to optimize the removal of phenolic compounds from sugarcane syrup and study the impact of the resulting purified syrup on the production process in yeast, in comparison to non-purified syrup, to disclose the effect of such compounds over this process. To achieve this, the specific objectives were to: i) optimize the removal process of phenolic compounds from the sugarcane syrup through adsorption with activated charcoal, according to a second-order polynomial model; ii) characterize the

purified syrup obtained under optimal conditions for its content in phenolic compounds, sugars and minerals and its antioxidant activity; iii) assess yeast growth, oxidative stress and production in batch fermentations, supplied with purified and non-purified syrup, using a recombinant *S. cerevisiae* strain producer of β-farnesene.

2. Materials and Methods

2.1.Sugarcane syrup purification

Activated charcoal was used in the optimization of sugarcane syrup purification, which was performed through an experimental design obtained from the software STATISTICA 14. A non-factorial central composite design (CCD) was performed according to the conditions described in Table 7. Adsorbent type (X_1), contact time (X_2) and adsorbent concentration (X_3) were studied. A total of 23 purifications were performed using 4 charcoal adsorbents from Sigma-Aldrich (Missouri, USA) and 1 from Proenol (Porto, Portugal): 100 mesh, 20-40 mesh, 12-20 mesh, 4-12 mesh and Proenol Carbon Pellet. These five types of the independent variable X_1 were coded from L1 to L5. Three levels of the independent variables X_2 and X_3 were tested and coded with -1, 0 or 1. Time of contact was 1, 12.5 or 24 h and adsorbent concentration was 10, 80 or 150 g of charcoal per L of syrup. The purifications were conducted in 250 mL Erlenmeyer flasks containing 100 mL of sugarcane syrup and the flasks were agitated at 100 rpm in an orbital incubator with 2.5 cm orbital diameter (New Brunswick Innova® 40, Eppendorf, Hamburg, Germany). After purification, the charcoal was separated from the syrup with centrifugation at 16435 *g* (Thermo Scientific Heraeus Multifuge X1R Centrifuge, Massachusetts, USA) for 10 min followed by two filtrations through a paper filter (Vreis, Lisbon, Portugal).

Table 7. Central composite design conditions for the optimization of sugarcane syrup purification with activated charcoal. L1: 100 mesh; L2: 20-40 mesh; L3: 12-20 mesh; L4: 4-12 mesh; L5: Proenol pellets.

Run	X_1 Adsorbent	X_2 Time of contact (h)	X_3 Adsorbent concentration (g/L)	Code X_1	Code X_2	Code X_3
1	L4	24.0	150	4	1	1
2	L4	24.0	10	4	1	-1
3	L2	24.0	150	2	1	1
4	L2	12.5	80	2	0	0
5	L5	1.0	150	5	-1	1
6	L2	24.0	10	2	1	-1
7	L1	24.0	10	1	1	-1
8	L5	12.5	10	5	0	-1
9	L3	12.5	80	3	0	0
10	L5	12.5	80	5	0	0
11	L3	1.0	10	3	-1	-1
12	L1	12.5	80	1	0	0
13	L5	24.0	80	5	1	0
14	L1	1.0	10	1	-1	-1
15	L2	1.0	150	2	-1	1
16	L3	1.0	150	3	-1	1
17	L5	1.0	10	5	-1	-1
18	L3	24.0	150	3	1	1
19	L4	1.0	10	4	-1	-1
20	L4	12.5	80	4	0	0
21	L1	1.0	150	1	-1	1
22	L3	24.0	10	3	1	-1
23	L2	12.5	80	2	0	0

The response surface model described in the second-order polynomial equation (7) was used to estimate the response variables. The X_1, X_2 and X_3 represent the independent variables and β_0 to β_9 represent the coefficient estimates. Analysis of variance and lack of fit tests were performed to assess the adequacy of the model.

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_1^2 + \beta_5 X_2^2 + \beta_6 X_3^2 + \beta_7 X_1 X_2 + \beta_8 X_1 X_3 + \beta_9 X_2 X_3 \qquad (7)$$

To graphically present the data, a multi-criteria predictive model was used according to equations (8), (9) and (10). Derringer's desirability function was used in the response

surface optimization (Suich and Derringer, 1980). Each answer variable's value was converted into a desirability score (d), which ranged from 0 to 1. Increased desirability is indicated by a higher score. A different optimization criterion (to maximize, minimize or obtain a fixed target) implies the use of a different equation. In these equations, *i* represents the individual response variables, $yi,_{min}$ and $yi,_{max}$ represent the minimum or maximum desired level of each response variable. A desirability score of 0 was given to responses below $yi,_{min}$ and a score of 1 was assigned to responses above $yi,_{max}$. A linear increase in desirability between $yi,_{min}$ and $yi,_{max}$ was obtained by assigning a weight (w_i) of 1. The adjustment of the response data was performed with STATISTICA 14.

$$d_i = 0, if y_i \ll y_{(i,min)} \quad (8)$$

$$d_i = \left[\left(\left(y_i - y_{(i,min)}\right)\right) / \left(\left(y_{(i,max)} - y_{(i,min)}\right)\right)\right]^{(w_i)} \quad (9)$$

$$d_i = 1, if y_i \gg y_{(i,max)} \quad (10)$$

After the purifications, the syrup was recovered and separated from the activated charcoal through centrifugation and filtration processes. During the purification procedure, some quantity of syrup remained in the materials used and was not recovered. Therefore, the syrup recovery yield (%) was calculated according to equation (11). For this calculation, the initial mass of syrup (g) before the process and the mass of syrup recovered (g) after the purification were used.

$$Syrup\ recovery\ yield = \frac{Mass\ of\ recovered\ syrup}{Mass\ of\ initial\ syrup}\ x\ 100 \quad (11)$$

2.2.Sugarcane syrup characterization

The Brazilian sugarcane syrup studied in this work, provided from Amyris Inc. USA, was characterized for its content of total reducing sugars (TRS) and phenolic compounds

before and after purification. The sugar content was determined by High Performance Liquid Chromatography (HPLC) with a Refractive Index Detector (RID) (Baptista et al., 2020). The compounds were separated in an Aminex HPX-87H column (300 x 7.8 mm) with a pre-column (30 x 4.6 mm) (Bio-rad, Hercules, USA). A mobile phase of 5 mM of sulfuric acid (Sigma-Aldrich, Missouri, USA) eluted through the column at 0.6 mL/min and 50 ˚C, while the RI detector (Agilent, Santa Clara, USA) was maintained at 35 ˚C. Concentrations ranging from 0.2 to 10 g/L of sucrose, glucose, and fructose standards (Sigma-Aldrich, Missouri, USA) were used to construct the respective calibration curves. TRS in the samples was expressed in g/L and calculated according to equation (12), where *S, G* and *F* represented the concentration of sucrose, glucose and fructose, respectively (Marques et al., 2015).

$$TRS\ (g/L) = \frac{S}{0.95} + G + F \qquad (12)$$

The identification and determination of all phenolic compounds were performed by LC-ESI-UHR-QqTOF-MS (Liquid Chromatography with Ultrahigh-Resolution, Electrospray Ionization, Quadrupole Time of Flight and Mass Spectrometry) (Bruker, Billerica, USA). The compound separation was performed in a Bruker Solo 2 C18 column (100 × 2.1 mm, 2.2 µm) (Bruker, Billerica, USA), with 0.1 % aqueous formic acid (mobile phase A) and acetonitrile with 0.1 % formic acid (mobile phase B) at a flow rate of 0.25 mL/min, and with a gradient according to Carvalho et al., 2022. Clusters of sodium formate were employed to calibrate the internal mass. The elemental composition and identity of phenolic compounds were validated by precise mass ([M-H]) and isotope rate estimates (mSigma). Standards of syringic acid, vanillic acid, 4-hydroxybenzoic acid, 3,4-dihydroxybenzalhedyde, 4-hydroxybenzaldehyde, gentisic acid, chlorogenic acid, ferulic acid, *p*-coumaric acid, caffeic acid, protocatechuic acid, luteolin, tricin, naringenin-7-*O*-glucoside (Sigma-Aldrich, Missouri, USA), isoschaftoside, orientin, diosmetin, vitexin, vitexin-2-*O*-rhamnoside and naringenin (Extrasynthése, Rhône, France) were used for the respective calibration curves. The total phenolic content (TPC) was calculated by the sum of all compounds detected and results were expressed in mg/L.

The chemical elements were analyzed in an Optima 7000 DV ICP-OES (inductively coupled plasma optical emission spectrometry; Perkin-Elmer, Massachusetts, USA)

according to the ICP methodology of Oliveira et al., 2022. Briefly, 200 µL of sugarcane syrup and 1.8 mL of ultrapure pure water were digested with 5 mL of nitric acid at 65 % (w/w; Sigma-Aldrich, Missouri, USA) and 2 mL of hydrogen peroxide at 30 % (w/w; CARLO ERBA, Emmendingen, Germany). Digestion occurred using a microwave-assisted digester XPERT (BERGHOF, Eningen, Germany) with a temperature and pressure reaching 190 ˚C and 40 bar for 10 min. The samples were diluted in ultrapure water to be analyzed in the ICP-OES equipment. Calibration curves of potassium, calcium, magnesium, phosphorous, sodium, molybdenum, iron, aluminum, manganese, and copper were used, and results were expressed in mg/L. Triplicate analyzes were performed for each sample.

The antioxidant activity of the sugarcane syrup was determined using the 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) method (Gonçalves et al., 2009). A solution with 1.92 g/L of ABTS and 0.33 g/L of potassium persulfate provided oxygen radicals to react with the sample and inhibition of ABTS radicals was measured. A calibration curve was constructed with trolox in a range of concentrations from 1.88 to 60 mg/L. For the antioxidant activity assay, 15 µL of the sample was added to 200 µL of the ABTS solution and stirred for 5 min at 30 ˚C. As a negative control, 15 µL of water was added to the ABTS solution. The absorbance was measured at 734 nm in a microplate reader (BioTek Synergy H1, Agilent, Santa Clara, USA). The ABTS inhibition, measured in percentage, was determined according to equation (13), where A_C is the absorbance obtained in the control and A_S is the absorbance obtained in the sample. Multiple sample dilutions were used to obtain an absorbance inside the calibration curve and, consequently, to determine the accurate ABTS inhibition.

$$ABTS\ Inhibition = \frac{A_C - A_S}{A_C}\ x\ 100 \quad (13)$$

The sample concentration required to obtain an antioxidant effect of 50 % (EC_{50}) was determined using the ABTS inhibition data. Multiple dilution experimental data with the ABTS inhibition and the sample concentration were plotted, and a trend line was constructed for each sample. The equation resulting from this trend line was used to determine the EC_{50} by assuming a 50 % inhibition in the equation.

2.3.Microorganism and inoculum

The yeast used in this work was a genetically engineered *S. cerevisiae* strain producing constitutively β-farnesene, provided by Amyris, Inc. Activation of the yeast was performed from two cryovials (with 1 mL of glycerol stock culture each) in a 250 mL Erlenmeyer flask with 63 mL of culture medium during 42 h of incubation. The second seed flask step was performed in 1 L Erlenmeyer flasks with 150 mL of culture medium, including 5.25 mL of inoculum from the first step. The medium of both seed cultures presented the same composition as in Carvalho et al., 2022, using sucrose as the carbon source. During 24 h of incubation, the seed flasks were agitated at 200 rpm and 30 °C in an incubator with 5 cm of orbital diameter (Eppendorf, New Brunswick™ Innova® 44).

2.4.Fermentation

The fermentations with purified and non-purified sugarcane syrups were performed in a batch mode both in shake-flasks and in bioreactors. A filtration system with 0.2 μm pores was used for syrup sterilization. The components of the medium were mostly the same as in the seed flask steps. However, a succinate buffer was not added, and the carbon source was provided by the sugarcane syrup at 80 g/L of TRS. The flask fermentations were performed in 1 L Erlenmeyer flasks, containing 150 mL of culture medium and the cell density was adjusted to start the culture with a washed optical density (wOD – measured at 600 nm) of 0.25. The flask cultures were maintained at 30 °C and 200 rpm until sugars were consumed by the yeast. Triplicate cultures were performed for each condition.

The bioreactor fermentations were performed in vessels with 2.7 L of working volume (Eppendorf, reference 76DR03F, Hamburg, Germany), with 1.5 L of culture medium and an initial wOD of 1. During the incubation time, a temperature of 30 °C, a pH of 5.0 (with the addition of 25 % ammonium hydroxide), aeration of 0.5 L/min and dissolved oxygen (DO) of at least 30 % were maintained. An agitation ramp ranging between 300 and 1200 rpm was used to provide DO higher than 30 %. Tergitol L-81 (Dow, Michigan, USA) was added at the start of the fermentation to avoid foam accumulation inside the reactors. Reactor fermentations were incubated until all sugars were consumed. Each condition was performed in duplicate.

2.5.Fermentation analysis

During the fermentation, yeast cell density was determined by measuring wOD of the fermentation broth, according to Carvalho et al., 2022. The absorbance was measured in a cuvette at 600 nm with a spectrophotometer (Shimadzu UV-1900 UV-VIS Spectrophotometer). The sugar consumption was determined during the fermentations by analyzing the sugar content in the supernatants of the fermentation broth. This was performed through HPLC according to the method described in section 2.2. The β-farnesene was quantified by Gas Chromatography (GC) with a Flame Ionization Detector (FID) and according to what is described in Carvalho et al., 2022 (McWilliam and Dewar, 1958). The extraction of β-farnesene from the fermentation broth was performed with a methanol solution and β-farnesene standards were used to construct the calibration curve. The concentration of β-farnesene was normalized to the last sampling time of the control condition.

Cell viability and reactive oxygen species (ROS) were also determined, through flow cytometry following the methodology of Lopes et al., 2023. For cell viability, the cells were incubated with a fluorescent probe, namely propidium iodide (Sigma-Aldrich, Missouri, USA). The fluorescence intensity was analyzed in the flow cytometer (BD Accuri™ C6 Plus Personal) with the fluorochrome phycoerythrin (PE). For intracellular ROS determination, the cells were incubated with the fluorescent probe dihydrorhodamine (Sigma-Aldrich, Missouri, USA). The fluorescence intensity was analyzed with the fluorochrome peridinin chlorophyll protein complex (Per-CP). The ROS levels were expressed in mean fluorescence units (MFU).

The kinetic parameters of the shake-flask and bioreactor fermentations were calculated. Equations 14 to 20 were used to determine substrate consumption (S), specific growth rate (μ), yield of biomass to substrate ($Y_{X/S}$), yield of product to substrate ($Y_{P/S}$), biomass productivity (P_X), substrate consumption rate (P_S) and product productivity (P_P), respectively. The S (in %) was calculated by using the initial substrate concentration (S_i, in g/L) and the current substrate concentration at the time of analysis (S_c, in g/L). To determine μ, in h^{-1}, the washed optical density at the time of analysis (wOD_c), the initial washed optical density (wOD_0), the current incubation time (t_c, in h) and the initial incubation time (t_0, in h) were used. For $Y_{X/S}$ (in g of biomass/ g of substrate consumed), the dry cell weight (DCW,

in g/L) and the substrate consumption (*S*, in g/L) were used. The $Y_{P/S}$ (g of product/g of substrate consumed) determined using the current product (*P*, in g/L) concentration at the time of analysis and *S*, in g/L. To determine P_X (in g/L/h), *DCW* and t_c were used. For P_S (in g/L/h), *S* and t_c were used. And for P_P (in g/L/h), *P* and t_c were used.

$$S = \frac{S_i - S_c}{S_i} \times 100 \quad (14)$$

$$\mu = \frac{\ln(wOD_c) - \ln(wOD_0)}{t_c - t_0} \quad (15)$$

$$Y_{X/S} = \frac{DCW}{S} \quad (16)$$

$$Y_{P/S} = \frac{P}{S} \quad (17)$$

$$P_X = \frac{DCW}{t_c} \quad (18)$$

$$P_S = \frac{S}{t_c} \quad (19)$$

$$P_P = \frac{P}{t_c} \quad (20)$$

2.6.Statistical analysis

The data obtained was examined with the statistical software STATISTICA 14. The normality of the data distribution was evaluated by the Shapiro-Wilk test. Student's *t*-test and one-way analysis of variance (ANOVA) with Fisher LSD post hoc tests were used to determine the statistical significance of a result. A *p*-value lower than 0.05 indicated statistically significant differences between conditions.

3. Results and discussion

3.1.Sugarcane syrup purification

3.1.1. Design of Experiment

To maximize the phenolic compounds removal from sugarcane syrup with activated charcoal, three independent variables were studied (Table 7): adsorption time (1, 12.5 and 24 h), adsorbent type (charcoal with 100, 20-40, 12-20 and 4-12 mesh or pellets) and adsorbent concentration (10, 80 and 150 g/L). The relationships between the response variables (phenolic compound purification and syrup recovery yield) and the independent variables were evaluated.

Like previously reported by our group (Carvalho et al., 2022), the LC-ESI-UHR-QqTOF-MS analysis for the phenolic profile of the sugarcane syrup resulted in the identification of 39 phenolic compounds (Annex Table 1). These compounds were grouped into three classes, namely hydroxybenzoic acids (HBA), hydroxycinnamic acids (HCA) and flavonoids. The main HBA compounds identified in the sugarcane syrup were hydroxybenzoic-4-β-glucoside and gentisic acid derivatives, at 5.0 and 9.0 mg/L, respectively. The most predominant HCA were *trans*-3-feruloylquinic acid and neochlorogenic acid, at 8.2 and 6.9 mg/L, respectively. Lastly, the flavonoids with higher concentrations were isoschaftoside at 1.6 mg/L and tricin-7-*O*-rhamnosyl-glucuronide at 1.3 mg/L. According to the polynomial second-order model, the concentrations of these compounds were compared in the syrup before and after purification with activated charcoal (Table 8). Results showed that HCA and flavonoids were removed in higher percentages from the syrup as compared to HBA. According to Richard et al., 2009, acidic phenolic compounds are absorbed by charcoal slower than non-acidic phenolic compounds, because of their different solubilities. This does not explain why the adsorption of the HBA and HCA classes, which are both phenolic acids, differs. Nonetheless, the research by Richard and co-authors supports the higher adsorption of flavonoids than the HBA class as it was found in our study.

Table 8. Total phenolic content (TPC) and phenolic classes (identified in LC-ESI-UHR-QqTOF-MS) concentration, syrup recovery yield and TPC purification obtained from the central composite design for the optimization of sugarcane syrup purification with activated charcoal. HBA: hydroxybenzoic acids. HCA: hydroxycinnamic acids.

Run	HBA (mg/L)	HBA Purification (%)	HCA (mg/L)	HCA Purification (%)	Flavonoids (mg/L)	Flavonoids Purification (%)	Syrup Yield (%)	TPC (mg/L)	TPC Purification (%)
Syrup	23.6 ± 3.3		45.6 ± 7.4		4.6 ± 1.6			73.4 ± 12.3	
1	9.0 ± 0.1	61.8 ± 0.1	7.6 ± 0.2	83.1 ± 0.2	0.4 ± 0.0	90.6 ± 0.0	21.9	17.1 ± 0.6	76.7 ± 0.3
2	23.6 ± 0.6	0.0 ± 0.6	47.8 ± 0.6	0.0 ± 0.0	5.2 ± 0.1	0.0 ± 0.0	61.0	76.7 ± 2.3	0.0 ± 1.4
3	2.3 ± 0.9	90.3 ± 0.9	0.3 ± 0.0	99.3 ± 0.0	0.0 ± 0.0	100.0 ± 0.0	19.4	2.6 ± 1.6	96.5 ± 1.0
4	11.2 ± 0.3	52.7 ± 0.3	11.4 ± 0.4	74.8 ± 0.4	0.7 ± 0.0	84.2 ± 0.0	42.2	23.3 ± 1.2	68.3 ± 0.7
5	10.5 ± 0.5	55.5 ± 0.5	1.8 ± 0.2	96.0 ± 0.2	0.0 ± 0.0	100.0 ± 0.0	39.8	12.3 ± 1.1	83.2 ± 0.7
6	19.7 ± 0.2	16.7 ± 0.2	40.4 ± 1.2	10.7 ± 1.2	6.5 ± 0.2	0.0 ± 0.0	78.2	66.5 ± 2.7	9.4 ± 1.6
7	15.3 ± 0.8	35.3 ± 0.8	14.5 ± 0.1	67.9 ± 0.1	2.0 ± 0.0	56.3 ± 0.0	49.5	31.8 ± 1.4	56.7 ± 0.8
8	20.3 ± 0.9	14.2 ± 0.9	25.7 ± 0.7	43.2 ± 0.7	1.0 ± 0.1	78.4 ± 0.1	63.9	46.9 ± 2.7	36.1 ± 1.6
9	12.9 ± 0.1	45.4 ± 0.1	30.2 ± 0.3	33.2 ± 0.3	1.2 ± 0.0	74.6 ± 0.0	53.9	44.3 ± 0.6	39.7 ± 0.4
10	6.2 ± 0.1	73.8 ± 0.1	0.0 ± 0.0	100.0 ± 0.0	0.0 ± 0.0	100.0 ± 0.0	39.6	6.2 ± 0.1	91.6 ± 0.1
11	23.8 ± 0.4	0.0 ± 0.0	45.6 ± 0.8	0.0 ± 0.0	4.4 ± 0.0	5.0 ± 0.0	82.2	73.8 ± 2.0	00.0 ± 0.0
12	14.4 ± 0.0	38.8 ± 0.0	23.6 ± 0.4	47.9 ± 0.4	2.1 ± 0.0	54.3 ± 0.0	49.0	40.1 ± 0.7	45.4 ± 0.4
13	7.1 ± 0.1	70.2 ± 0.1	0.0 ± 0.0	100.0 ± 0.0	0.0 ± 0.0	100.0 ± 0.0	24.8	7.1 ± 0.2	90.4 ± 0.1
14	17.1 ± 0.0	27.4 ± 0.0	32.9 ± 0.2	27.3 ± 0.2	2.7 ± 0.0	42.1 ± 0.0	83.2	52.7 ± 0.2	28.2 ± 0.1
15	11.3 ± .2	52.2 ± 0.2	15.4 ± 0.1	66.0 ± 0.1	1.3 ± 0.0	72.5 ± 0.0	33.7	27.9 ± 0.2	62.0 ± 0.1
16	14.9 ± 0.0	37.0 ± 0.0	26.7 ± 0.0	40.9 ± 0.0	2.5 ± 0.0	44.7 ± 0.0	37.2	44.1 ± 0.1	39.9 ± 0.0
17	19.6 ± 0.1	17.2 ± 0.1	38.1 ± 0.5	15.7 ± 0.5	3.6 ± 0.0	21.9 ± 0.0	49.6	61.2 ± 1.1	16.6 ± 0.6
18	6.2 ± 0.0	73.7 ± 0.0	2.1 ± 0.0	95.4 ± 0.0	0.1 ± 0.0	96.8 ± 0.0	25.0	8.4 ± 0.0	88.5 ± 0.0
19	23.2 ± .2	1.9 ± 0.2	45.5 ± 0.2	0.0 ± 0.0	4.3 ± 0.0	6.8 ± 0.0	80.9	72.9 ± 0.7	0.7 ± 0.4
20	3.6 ± 0.0	84.6 ± 0.0	0.0 ± 0.0	100.0 ± 0.0	0.0 ± 0.0	100.0 ± 0.0	24.1	3.6 ± 0.0	95.0 ± 0.0
21	3.1 ± 0.1	86.8 ± 0.1	0.0 ± 0.0	100.0 ± 0.0	0.0 ± 0.0	100.0 ± 0.0	34.2	3.1 ± 0.1	95.8 ± 0.1
22	20.5 ± 0.5	13.2 ± 0.5	41.4 ± 0.8	8.3 ± 0.8	4.0 ± 0.1	13.9 ± 0.1	76.0	65.9 ± 2.2	10.2 ± 1.3
23	10.0 ± 0.1	57.7 ± 0.1	8.2 ± 0.1	81.9 ± 0.1	0.5 ± 0.0	89.6 ± 0.0	47.2	18.6 ± 0.4	74.6 ± 0.3

The maximum purification was obtained in run 3 (L2 charcoal at 150 g/L and 24 h of contact), in which 96.5 % of TPC was removed from the syrup (Table 8). On the other hand, the highest syrup recovery yield was obtained in run 14 (L1 charcoal at 10 g/L and 1 h of contact), in which 83.2 % of the syrup mass was recovered after the purification process. In fact, the regression plot between syrup purification and syrup recovery yield (Figure 12) showed that these two runs were at the opposite ends of the graph: in run 3 great purification was observed, but only 19.4 % of syrup was recovered; while in run 14 high yield was obtained, but only 28.2 % of TPC was removed from the syrup. These data suggested that both dependent variables were inversely related.

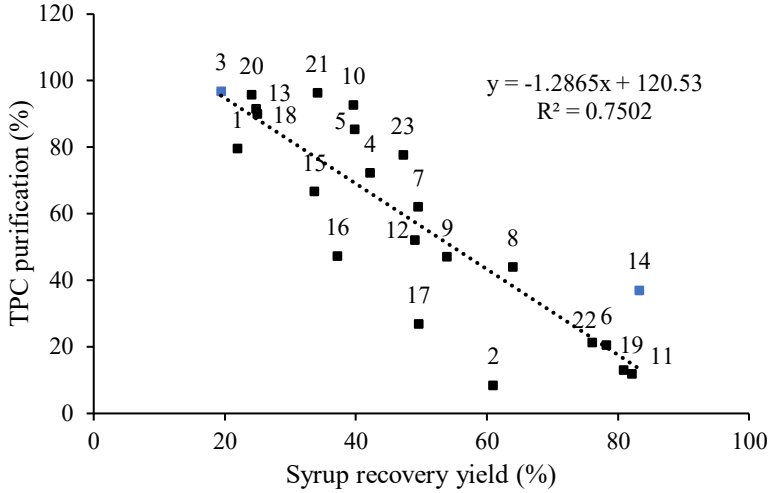

Figure 12. Relationship between phenolic purification and syrup recovery yield resulting from the 23 runs of central composite design for the optimization of sugarcane syrup purification. TPC – total phenolic content. The number of each data label represents the run.

The results presented in Table 8 were plotted in Pareto charts to evaluate the effect of the operational conditions in the syrup purification process and the standardized effects are presented in Figure 13. It was found that higher quantities of charcoal (X_3) led to increased adsorption of phenolic compounds from the syrup ($p < 0.0001$; Figure 13A), due to the availability of increased mass of charcoal for adsorption. However, this operational condition with more charcoal decreased the yields of syrup recovery ($p < 0.0001$; Figure 13B). Since the syrup had high viscosity, its separation from the adsorbent with a low particle size (L1 at 0.149 mm compared to L5 at 4.76 mm) became more difficult especially for higher quantities of adsorbent. Additionally, in the TPC purification variable, the quadratic term of the adsorbent type (X_1^2) also presented a significant effect (Figure 13A). The contact time (X_2) between the charcoal and the syrup (X_2, $p > 0.05$) did not show any effect on syrup purification or yield, with no significant increase in purification observed between 1 and 24 h of incubation.

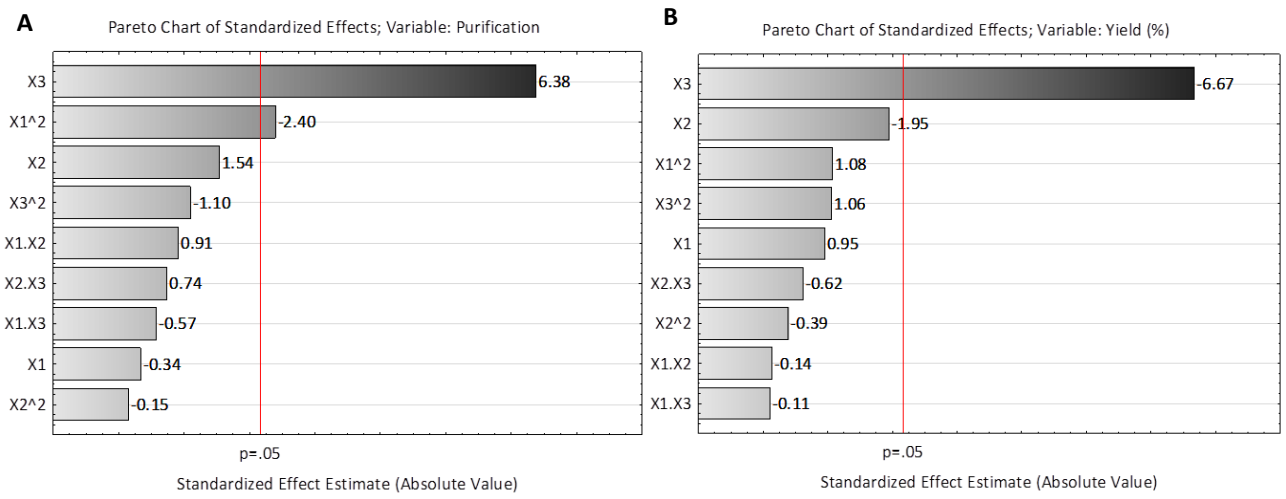

Figure 13. Pareto charts of standardized effects of central composite design for the optimization of sugarcane syrup purification. A: purification of phenolic compounds (%); B – syrup recovery yield (%). Bars past the line represent statistically significant results ($p < 0.05$). X_1 – adsorbent type; X_2 – contact time; X_3 – adsorbent concentration.

According to many works, activated charcoal has been used to remove phenolic compounds from several types of biomasses. For instance, Lu et al., 2013 have found that 99.9 % of phenolic compounds were removed from wood pulping hydrolysate when using 2 % (w/v) of charcoal at pH 2.0, at 90 ºC and stirred for 30 min. Additionally, in the work by Preechakun et al., 2022, 77 % of phenolic compounds were removed from a sugarcane bagasse hydrolysate with 10 % (w/v) of activated charcoal at 25 ºC, stirred for 4 h and without pH adjustment. While the study of Lu and co-workers was able to remove almost completely all the phenolic compounds using only 2 % (w/v) of charcoal, the pH was adjusted to 2.0, which requires the use of an acid during purification and, subsequently, the purified syrup needs to be neutralized (by addition of base) to be used in a fermentation. In addition, extra thermal energy is required to maintain a temperature of 90 ºC. This procedure undoubtedly raises the price of the purifying process. On the other hand, in the study by Preechakun et al., 2022 a removal of 77 % phenolic compounds from sugarcane bagasse hydrolysate by using 10 % (w/v) of charcoal was obtained. In the current experiment, the application of 15 % (w/v) charcoal at room temperature resulted in 96.5% purification of phenolic compounds (run 3), which was 19 % higher than the purification percentage from the sugarcane bagasse obtained by Preechakun et al., 2022.

3.1.2. Validation of the predicted purification conditions and characterization of sugarcane syrup

The statistical analysis of the central composite design indicated that the proposed model was adequate ($R^2 = 0.82$) to describe the best purification conditions for obtaining a syrup with fewer phenolic compounds. The predictive model was applied to the data (Table 8) to discover the purification parameters that would result in a syrup with the least concentration of phenolic compounds and the most syrup mass at the end of the procedure. Because it was more important for this study to remove phenolic compounds rather than recover as much syrup as possible, the purification variable was given double the importance over the syrup recovery yield. The model predicted, with a desirability of 0.76, that the best purification condition would be obtained with the charcoal pellets from Proenol (L5) at a concentration of 115 g/L with 12.5 h of contact time. This condition predicted a TPC purification response of 93.62 ± 17.66 % and a syrup recovery yield of 31.50 ± 12.03 %. Among the other charcoals (L1 to L4), the main difference was the particle size, in which L1 presented the smallest (at 100 mesh) and L4 the largest (at 4 to 12 mesh) particle size. This meant that the surface area of the smallest charcoal (L1 had around 1150 m^2/g) was higher than that of the largest charcoal (L4 with around 700 m^2/g). In fact, Deng et al., 2018 found that the charcoals with lower particle size were able to adsorb more molecules. However, the L5 charcoal used in our study presented a higher overall particle size but also contained a higher surface area than the other charcoals (around 1350 m^2/g). Therefore, for the same mass of activated charcoal, the L5 likely had more surface points to which the phenolic compounds could be adsorbed than the other charcoals, leading to higher adsorption and removal from the syrup.

Considering the amount of charcoal employed, the elimination of phenolic compounds was greater when higher content of adsorbent was used (Figure 14). A similar elimination rate of phenolic compounds was reported by Deng et al., 2018, in which sugarcane bagasse hydrolysates were treated with 12.6 % (w/w) of activated charcoal and 98 % of phenolic compounds were removed. However, in the current work, less syrup was recovered when high quantity of charcoal was used. Therefore, the predicted optimal point was a compromise between both dependent variables, in which it was expected to obtain great purification with optimal syrup recovery yield.

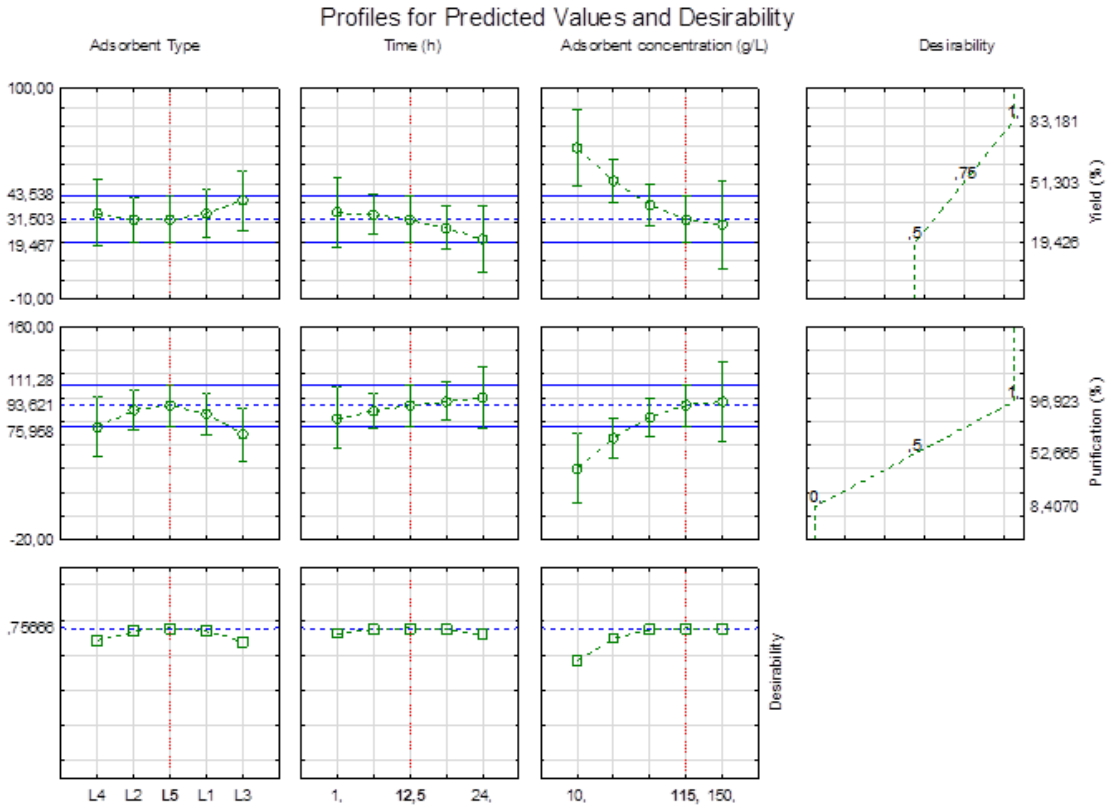

Figure 14. Profiles for predicted values and desirability of central composite design for the optimization of sugarcane syrup purification with activated charcoal. Red dashed lines represent the predicted optimal condition.

To validate the predictive model, three purifications were performed under the optimal predicted conditions (Proenol pellets at 115 g/L of syrup and 12.5 h of contact time), and the results are presented in Table 9. Results showed that, at this optimal condition, the TPC decreased by 96.73 ± 0.14 % and the syrup recovery yield was 43.65 ± 1.20 %. The predicted values (TPC purification of 93.62 ± 17.66 % and syrup recovery yield of 31.50 ± 12.03 %) agreed with the experimental values ($p > 0.05$ using a paired *t*-test). Therefore, the predictive performance of the established model can be considered validated and most phenolic compounds can be efficiently removed from the syrup with this purification condition.

Table 9. Phenolic content, sugar concentration, mineral content and antioxidant activity of non-purified and purified sugarcane syrup at optimal condition from central composite design: with charcoal pellets from Proenol at 115 g/L and agitated during 12.5 h. Each value represents the average from triplicates and the errors represent the standard deviation. 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. ND: not detected.

Parameter group	Parameter	Non-purified syrup	Purified syrup
Phenolic content (mg/L)	HBA	23.6 ± 3.3 (a)	2.4 ± 0.1 (b)
	HCA	45.2 ± 7.4	ND
	Flavonoids	4.6 ± 1.6	ND
	TPC	73.4 ± 12.3 (a)	2.4 ± 0.1 (b)
Sugar content (g/L)	Sucrose	396.3 ± 1.1 (a)	413.9 ± 0.8 (b)
	Fructose	165.2 ± 0.6 (a)	174.6 ± 0.3 (b)
	Glucose	100.1 ± 0.5 (a)	105.2 ± 0.1 (b)
	TRS	682.4 ± 2.3 (a)	715.5 ± 1.1 (b)
Mineral content (mg/L)	Potassium	6212.88 ± 113.66 (a)	5965.54 ± 269.30 (a)
	Calcium	1340.35 ± 131.71 (a)	1395.87 ± 124.01 (a)
	Magnesium	777.43 ± 58.32 (a)	953.81 ± 88.40 (a)
	Phosphorous	97.52 ± 13.69 (a)	513.86 ± 50.39 (b)
	Sodium	65.97 ± 38.97 (a)	382.49 ± 39.25 (b)
	Molybdenum	14.86 ± 0.00 (a)	14.36 ± 0.26 (a)
	Iron	14.08 ± 2.08	ND
	Aluminum	9.42 ± 0.03 (a)	4.45 ± 0.55 (b)
	Manganese	7.72 ± 0.95 (a)	6.12 ± 0.67 (a)
	Copper	0.54 ± 0.06 (a)	0.11 ± 0.04 (b)
Antioxidant activity	ABTS EC_{50} (mL/mL)	0.019 ± 0.001 (a)	0.147 ± 0.009 (b)

The purification of sugarcane syrup at the optimal condition resulted in the complete removal of 35 phenolic compounds, including molecules with high inhibitory activity over the yeast (Gu et al., 2015; Merkl et al., 2010) from the HBA class (1.28 mg/L of protocatechuic acid and 2.15 mg/L of 4-hydroxybenzaldehyde) and the HCA class (5.53 mg of *p*-coumaric acid, 2.65 mg/L of ferulic acid and 1.12 mg/L of caffeic acid). In fact, no HCA or flavonoids were detected in the purified sugarcane syrup. Therefore, the only class of phenolic compounds that remained after purification was HBA, at 10 % of the concentration found in the non-purified syrup (reduced from 23.6 in non-purified syrup to 2.4 mg/L in purified). Specifically, the compounds remaining in the syrup were hydroxybenzoic-4-β-

glucoside at 1.12 mg/L, gentisic acid 2-*O*-β-glucoside at 0.52 mg/L, and gentisic acid 5-*O*-β-glucoside at 0.72 mg/L.

The purified sugarcane syrup was also characterized regarding its sugar content and compared with non-purified syrup (Table 9). It was found that the purification process resulted in an increase of the sugars present in the syrup by 5 %. Since charcoal has been shown to have a higher affinity for the phenolic compounds than for the sugars (Bernal et al., 2016; Lee and Park, 2016), it was expected that sugars would not be removed from the syrup together with the charcoal. The syrup was filtered overnight inside a laminar flow chamber to allow maximum charcoal separation and simultaneous sterilization. Hence, during the purification and removal of the charcoal, water from the syrup may have evaporated, thus concentrating the sugar.

Because the EC_{50} values of the ABTS in the purified syrup were much greater than in the non-purified syrup, the antioxidant activity of the syrup decreased with purification (Table 9). The EC_{50}, or the concentration required to reduce antioxidant activity by 50%, increased 6.7-fold. These results were expected, due to the removal of phenolic compounds that contributes to the antioxidant activity (Carvalho et al., 2021). Previous studies showed that the removal of TPC from cashew leaf extracts with activated charcoal decreased the antioxidant activity of the purified biomaterial from 50 to 80 % (Chermahini et al., 2011), which agrees with the results obtained in the current work.

Chemical components such as calcium, magnesium, and potassium are also present in sugarcane syrup (Thai and Doherty, 2011), which can be used by the yeast during the fermentation process. Therefore, to evaluate if the purification with charcoal affected the mineral composition of sugarcane syrup, the chemical elements were analyzed through the ICP methodology (Table 9). The main elements found in the syrup were potassium, calcium and magnesium and their concentrations did not change with the removal of phenolic compounds. However, the purification process led to an increase of phosphorous and sodium in the syrup by 4.3 and 4.8-fold, respectively. The charcoal pellets from Proenol were activated with phosphoric acid, which may explain the increase of phosphorous in the syrup. Furthermore, the purification also produced a syrup with lower quantities of iron, aluminum, and copper. Iron, an element required for cell respiration, was completely removed from the syrup and was described to have affinity to activated charcoal (Nazir et al., 2021; Shakoury-Elizeh et al., 2010). Regarding aluminum, its content decreased by 53 %, and Pour et al.,

2014 showed that activated charcoal binds to this element in aqueous solutions. Furthermore, Abdulrazak et al., 2017 reported that copper has affinity to activated charcoal and, in the current work, the copper content of syrup decreased by 80 %. Because iron, aluminum, and copper have a high affinity for activated charcoal, the purifying method reduced their levels in sugarcane syrup.

In the literature, the elemental composition of sugarcane syrup has not been reported. However, sugarcane juice (syrup before the evaporation step) was analyzed for its content in several elements (Souza et al., 2019; Sperança et al., 2021). In these works, the concentrations of phosphorous, calcium and magnesium were found to be between 5 to 10 times lower than what was found in the sugarcane syrup in our work. This result may be expected, due to the evaporation of the juice that occurs to produce the syrup, which concentrates all components by 2.5 to 4 times (Vicentini-Polette et al., 2019).

3.2.Fermentation with purified syrup as feedstock

To assess the impact of removing phenolic compounds from sugarcane syrup on the yeast fermentation process, cultures were performed at the shake-flask and bioreactor scales using the purified syrup (TPC of 0.25-0.29 mg/L) and non-purified syrup (TPC of 6.23-7.08 mg/L) described in section 3.1.2. In shake-flasks, no difference was detected in TRS consumption, as shown in Figure 15, while cell optical density and β-farnesene concentration at the end of the fermentation were 16 % and 12 % higher, respectively, with purified syrup. Cell viability was above 95 % during the entire fermentation for both conditions (Figure 15C). Total ROS was similar at the beginning of the fermentation but presented significant differences during the incubation time (Figure 15D). In the exponential phase, total ROS was 79 % higher in the presence of purified syrup, while in the stationary phase this difference was 20 %.

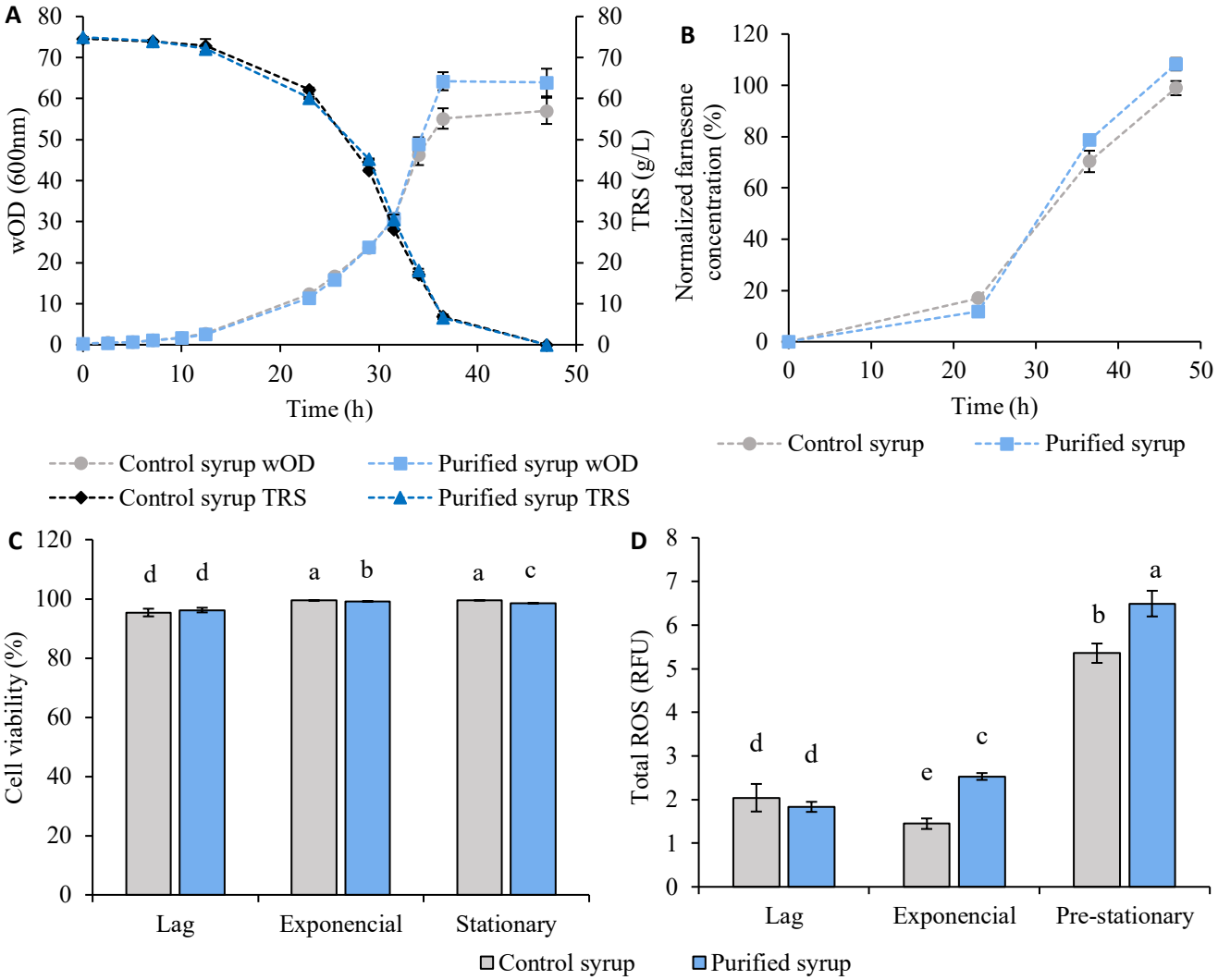

Figure 15. Washed optical density (wOD) measured at 600 nm and total reducing sugars (TRS; A), β-farnesene concentration normalized to the last sample of control (B), cell viability (C) and total reactive oxygen species (ROS; D) in the lag, exponential and pre-stationary phases during *S. cerevisiae* shake-flask batch fermentations using media with non-purified or purified sugarcane syrups as the carbon source. RFU: relative fluorescent unit. Each value represents the average of three replicate shake flask fermentations and the errors represent the standard deviation. Different letters represent the significant differences from a *t*-test (n = 3) between conditions.

In bioreactors, as shown in Figure 16, in the presence of purified syrup an increase in final cell density and β-farnesene concentration of 7 % and 9 %, respectively, was obtained. However, the fermentation with the purified syrup started with 8 % more sugars, which could justify these increases in biomass and product. During the incubation, cell viability remained above 98 % for both conditions. Total ROS in the exponential and pre-stationary phases presented a significant rise of 22 % and 49 %, respectively when purified syrup was used.

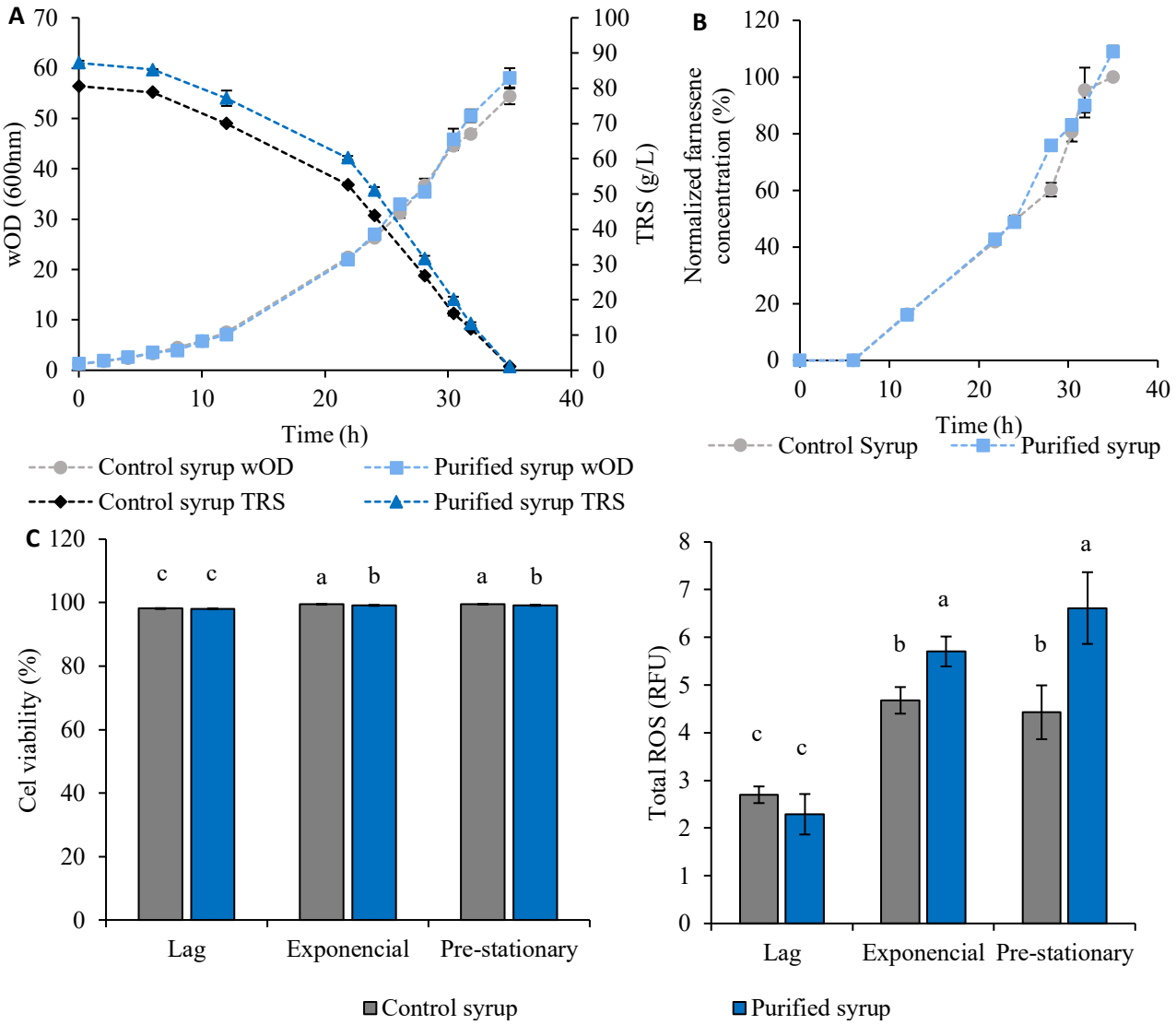

Figure 16. Washed optical density (wOD) measured at 600 nm and total reducing sugars (TRS; A), β-farnesene concentration normalized to the last sample of control (B), cell viability (C) and total reactive oxygen species (ROS; D) in the lag, exponential and pre-stationary phases during *S. cerevisiae* bioreactor batch fermentation using media with non-purified or purified sugarcane syrups as the carbon source. RFU: relative fluorescent unit. Each value represents the average of two replicate bioreactor fermentations and the errors represent the standard deviation. Different letters represent the significant differences from a *t*-test (n = 3) between conditions.

When comparing the shake-flask and bioreactor fermentations (Figure 15D and Figure 16D), the yeast cells in the reactor showed an increase in ROS of 34 % when using purified syrup and 9 % when using control syrup, indicating the existence of increased oxidative stress in the bioreactor process. These results demonstrate that the phenolic compounds presence in sugarcane syrup may represent an advantage by conferring higher oxidative stability during fermentation.

In Table 10, the fermentation parameters of both the shake-flask and the bioreactor fermentations are presented. Results showed that, in shake-flasks, the specific growth rate (μ) was higher in the presence of purified syrup, while the yield of product to substrate ($Y_{P/S}$) increased by 11 %. Additionally, the β-farnesene productivity (P_P) obtained in fermentations with purified syrup was 11 % higher in shake-flasks. In bioreactors, the yield of biomass to substrate ($Y_{X/S}$) increased by 3 % and the biomass productivity (P_X) increased by 12 % with the purification of syrup. On the other hand, the substrate consumption rate (P_S) was also 8 % higher in purified syrup in bioreactor fermentations.

Table 10. Fermentation parameters of β-farnesene shake-flask and bioreactor cultures by *S. cerevisiae* with non-purified and purified sugarcane syrups after 35 h of culture. TPC - total phenolic content.

Condition	TPC (mg/L)	μ (h^{-1})	S (%)	$Y_{X/S}$ (g/g)	$Y_{P/S}$ (g/g)	P_X (g/L/h)	P_S (g/L/h)	P_P (g/L/h)
Shake-flask with non-purified syrup*	6.23	$0.144^{b} \pm 0.000$	$90.5^{b} \pm 0.8$	$0.307^{a} \pm 0.003$	$0.123^{c} \pm 0.005$	$0.594^{ab} \pm 0.002$	$1.93^{c} \pm 0.02$	$0.238^{c} \pm 0.009$
Shake-flask with purified syrup**	0.25	$0.146^{a} \pm 0.001$	$91.4^{b} \pm 0.4$	$0.295^{a} \pm 0.005$	$0.136^{b} \pm 0.001$	$0.577^{b} \pm 0.001$	$1.96^{c} \pm 0.01$	$0.265^{b} \pm 0.004$
Bioreactor with non-purified syrup*	7.08	$0.109^{c} \pm 0.000$	$95.5^{a} \pm 0.1$	$0.240^{c} \pm 0.001$	$0.157^{a} \pm 0.001$	$0.543^{c} \pm 0.000$	$2.26^{b} \pm 0.01$	$0.354^{a} \pm 0.014$
Bioreactor with purified syrup**	0.29	$0.110^{c} \pm 0.000$	$95.6^{a} \pm 0.1$	$0.248^{b} \pm 0.002$	$0.154^{a} \pm 0.001$	$0.608^{a} \pm 0.000$	$2.45^{a} \pm 0.02$	$0.377^{a} \pm 0.010$

* Non-purified syrup: mixture of 39 phenolic compounds with TPC of 73.4 mg/L.

** Purified syrup: mixture of 5 phenolic compounds at TPC of 2.4 mg/L (hydroxybenzoic-4-β-glucoside at 1.12 mg/L, gentisic acid 2-*O*-β-glucoside at 0.52 mg/L, and gentisic acid 5-*O*-β-glucoside at 0.72 mg/L).

Overall, in the presence of purified syrup, the yeast presented better growth rate in shake-flasks and biomass yield in bioreactors. Substrate consumption also increased in bioreactors with fewer phenolic compounds. Moreover, the use of purified syrup also influenced the production parameters in shake-flasks, in which there was an increase in both $Y_{P/S}$ and P_P. Oxidative stress, indicated by the total ROS levels, was also generally higher with purified syrup in both shake-flasks and reactors. Therefore, the reduction of phenolic compounds can be associated to better growth and higher oxidative stress, which is certainly due to the removal of molecules with antimicrobial and antioxidant effects. The effects of phenolic compounds on *S. cerevisiae* have been widely reported (Adeboye et al., 2014; Cunha et al., 2018; Gu et al., 2019; Larsson et al., 2000). The presence of certain phenolic compounds, such as flavonoids or ferulic, cinnamic, caffeic and coumaric acids, was

reported to decrease the oxidative stress (Ronen et al., 2013; Tungmunnithum et al., 2022). On the other hand, the presence of antimicrobial phenolic compounds, such as 4-hydroxybenzaldehyde, have been correlated with lower ethanol productivity in *S. cerevisiae* in shake-flask fermentation (Gu et al., 2019; Larsson et al., 2001). Similarly, lower β-farnesene productivity (P_P) was seen in the current work at shake-flask scale with non-purified syrup, as compared to purified syrup (in which 4-hydroxybenzaldehyde and other inhibitory compounds were totally removed). However, in bioreactors, the production parameters were not changed with the removal of phenolic compounds. This was different from what was obtained in shake-flasks, where the production improved with purified syrup. Adeboye et al., 2015 have reported that the difference in yeast performance between two different scales may be attributed to better agitation, aeration, and pH control at the reactor scale. In fact, in the current work, the fermentations performed at the reactor scale were controlled to maintain the dissolved oxygen at a minimum of 30 % and a pH of 5.0. In shake-flasks, despite the initial pH also being 5.0, there was no control over these two parameters during the fermentation. Increased oxidative stress, as seen in the bioreactors, has also been reported to deviate the yeast metabolism towards antioxidant-related mechanisms, which could lower the productivity (Auesukaree, 2017). This oxidative stress could then justify the similar P_P in bioreactors with purified and non-purified syrups, as opposed to the higher P_P found in shake-flasks with purified syrup. Thus, this study reveals that the scale of fermentation determines how phenolic compounds from sugarcane syrup influence the yeast fermentation, with bioreactors offering a better control over the fermentation environment.

4. Conclusions

For the first time, phenolic compounds were removed from sugarcane syrup by using activated charcoal. Charcoal with high surface area (~1350 m^2/g) at 115 g/L was able to absorb 97 % of phenolic compounds contained in the syrup, significantly decreasing syrup antioxidant activity, leading to higher oxidative stress in the fermentation. Syrup purification enhanced β-farnesene productivity by 12 % in shake-flasks, while this parameter did not change in 2 L batch bioreactor, although yeast growth was improved. Thus, 7.08 mg/L of phenolic mixture can impact on yeast productivity at small-scale but not at large-scale, for the process and conditions tested.

References

Abdulrazak, S., Hussaini, K., Sani, H.M., 2017. Evaluation of removal efficiency of heavy metals by low-cost activated carbon prepared from African palm fruit. Appl Water Sci 7, 3151–3155. https://doi.org/10.1007/s13201-016-0460-x

Adeboye, P.T., Bettiga, M., Aldaeus, F., Larsson, P.T., Olsson, L., 2015. Catabolism of coniferyl aldehyde, ferulic acid and p-coumaric acid by Saccharomyces cerevisiae yields less toxic products. Microb Cell Fact 14, 149. https://doi.org/10.1186/s12934-015-0338-x

Adeboye, P.T., Bettiga, M., Olsson, L., 2014. The chemical nature of phenolic compounds determines their toxicity and induces distinct physiological responses in Saccharomyces cerevisiae in lignocellulose hydrolysates. AMB Express 4, 46. https://doi.org/10.1186/s13568-014-0046-7

Almeida, J.R.M., Modig, T., Petersson, A., Hähn-Hägerdal, B., Lidén, G., Gorwa-Grauslund, M.F., 2007. Increased tolerance and conversion of inhibitors in lignocellulosic hydrolysates by Saccharomyces cerevisiae. Journal of Chemical Technology & Biotechnology 82, 340–349. https://doi.org/10.1002/jctb.1676

Auesukaree, C., 2017. Molecular mechanisms of the yeast adaptive response and tolerance to stresses encountered during ethanol fermentation. J Biosci Bioeng 124, 133–142. https://doi.org/https://doi.org/10.1016/j.jbiosc.2017.03.009

Baptista, S.L., Carvalho, L.C., Romaní, A., Domingues, L., 2020. Development of a sustainable bioprocess based on green technologies for xylitol production from corn cob. Ind Crops Prod 156. https://doi.org/10.1016/j.indcrop.2020.112867

Bernal, M., Ruiz, M.O., Geanta, R.M., Benito, J.M., Escudero, I., 2016. Colour removal from beet molasses by ultrafiltration with activated charcoal. Chemical Engineering Journal 283, 313–322. https://doi.org/https://doi.org/10.1016/j.cej.2015.07.047

Canilha, L., Carvalho, W., de Almeida Felipe, M.D.G., de Almeida E Silva, J.B., Giulietti, M., 2010. Ethanol production from sugarcane bagasse hydrolysate using Pichia stipitis. Appl Biochem Biotechnol 161, 84–92. https://doi.org/10.1007/S12010-009-8792-8/TABLES/3

Carvalho, L.C., Oliveira, A.L.S., Carsanba, E., Pintado, M., Oliveira, C., 2022. Phenolic compounds modulation in β-farnesene fed-batch fermentation using sugarcane syrup as feedstock. Ind Crops Prod 188, 115721. https://doi.org/10.1016/J.INDCROP.2022.115721

Carvalho, M.J., Oliveira, A.L., Pedrosa, S.S., Pintado, M., Madureira, A.R., 2021. Potential of sugarcane extracts as cosmetic and skincare ingredients. Ind Crops Prod 169, 113625. https://doi.org/10.1016/J.INDCROP.2021.113625

Chermahini, S.H., Adibah, F., Majid, A., Sarmidi, M.R., 2011. Antioxidant properties of cashew leaves extracts before and after treatment with activated carbon used in cosmetics. Journal of Medicinal Plants Research 5, 4162–4170. https://doi.org/10.5897/JMPR.9000226

Cunha, J.T., Romaní, A., Costa, C.E., Sá-Correia, I., Domingues, L., 2018. Molecular and physiological basis of Saccharomyces cerevisiae tolerance to adverse lignocellulose-based process conditions. Applied Microbiology and Biotechnology 2018 103:1 103, 159–175. https://doi.org/10.1007/S00253-018-9478-3

Deng, F., Cheong, D.Y., Aita, G.M., 2018. Optimization of activated carbon detoxification of dilute ammonia pretreated energy cane bagasse enzymatic hydrolysate by response surface methodology. Ind Crops Prod 115, 166–173. https://doi.org/10.1016/J.INDCROP.2018.02.030

Duarte-Almeida, J.M., Salatino, A., Genovese, M.I., Lajolo, F.M., 2011. Phenolic composition and antioxidant activity of culms and sugarcane (Saccharum officinarum L.) products. Food Chem 125, 660–664. https://doi.org/https://doi.org/10.1016/j.foodchem.2010.09.059

Gonçalves, B., Falco, V., Moutinho-Pereira, J., Bacelar, E., Peixoto, F., Correia, C., 2009. Effects of elevated CO2 on grapevine (Vitis vinifera L.): volatile composition, phenolic content, and in vitro antioxidant activity of red wine. J Agric Food Chem 57, 265–273. https://doi.org/10.1021/JF8020199

Gu, H., Zhang, J., Bao, J., 2015. High tolerance and physiological mechanism of Zymomonas mobilis to phenolic inhibitors in ethanol fermentation of corncob residue. Biotechnol Bioeng 112, 1770–1782. https://doi.org/10.1002/bit.25603

Gu, H., Zhu, Y., Peng, Y., Liang, X., Liu, X., Shao, L., Xu, Y., Xu, Z., Liu, R., Li, J., 2019. Physiological mechanism of improved tolerance of Saccharomyces cerevisiae to lignin-derived phenolic acids in lignocellulosic ethanol fermentation by short-term adaptation. Biotechnol Biofuels 12, 268. https://doi.org/10.1186/s13068-019-1610-9

Guil-Guerrero, J.L., Ramos, L., Moreno, C., Zúñiga-Paredes, J.C., Carlosama-Yepez, M., Ruales, P., 2016. Antimicrobial activity of plant-food by-products: A review focusing on the tropics. Livest Sci 189, 32–49. https://doi.org/10.1016/J.LIVSCI.2016.04.021

Hill, P., Benjamin, K., Bhattacharjee, B., Garcia, F., Leng, J., Liu, C.L., Murarka, A., Pitera, D., Rodriguez Porcel, E.M., da Silva, I., Kraft, C., 2020. Clean manufacturing powered by biology: how Amyris has deployed technology and aims to do it better. J Ind Microbiol Biotechnol 47, 965–975. https://doi.org/10.1007/S10295-020-02314-3

Huang, X., Zhu, G., Liu, Q., Chen, L., Li, Y., Hou, B., 2018. Modulation of Plant Salicylic Acid-Associated Immune Responses via Glycosylation of Dihydroxybenzoic Acids1. Plant Physiol 176, 3103–3119. https://doi.org/10.1104/pp.17.01530

Hur, S.J., Lee, S.Y., Kim, Y.-C., Choi, I., Kim, G.-B., 2014. Effect of fermentation on the antioxidant activity in plant-based foods. Food Chem 160, 346–356. https://doi.org/10.1016/j.foodchem.2014.03.112

Joannis-Cassan, C., Riess, J., Jolibert, F., Taillandier, P., 2014. Optimization of very high gravity fermentation process for ethanol production from industrial sugar beet syrup. Biomass Bioenergy 70, 165–173. https://doi.org/10.1016/J.BIOMBIOE.2014.07.027

Juan, C.A., de la Lastra, J.M.P., Plou, F.J., Pérez-Lebeña, E., 2021. The Chemistry of Reactive Oxygen Species (ROS) Revisited: Outlining Their Role in Biological Macromolecules (DNA, Lipids and Proteins) and Induced Pathologies. International Journal of Molecular Sciences 2021, Vol. 22, Page 4642 22, 4642. https://doi.org/10.3390/IJMS22094642

Kamal, S.M.M., Mohamad, N.L., Abdullah, A.G.L., Abdullah, N., 2011. Detoxification of sago trunk hydrolysate using activated charcoal for xylitol production. Procedia Food Sci 1, 908–913. https://doi.org/10.1016/J.PROFOO.2011.09.137

Kim, Y., Kreke, T., Hendrickson, R., Parenti, J., Ladisch, M.R., 2013. Fractionation of cellulase and fermentation inhibitors from steam pretreated mixed hardwood. Bioresour Technol 135, 30–38. https://doi.org/10.1016/j.biortech.2012.10.130

Larsson, S., Nilvebrant, N.-O., Jönsson, L., 2001. Effect of overexpression of Saccharomyces cerevisiae Pad1p on the resistance to phenylacrylic acids and lignocellulose hydrolysates under aerobic and oxygen-limited conditions. Appl Microbiol Biotechnol 57, 167–174. https://doi.org/10.1007/s002530100742

Larsson, S., Quintana-Sáinz, A., Reimann, A., Nilvebrant, N.-O., Jönsson, L.J., 2000. Influence of Lignocellulose-Derived Aromatic Compounds on Oxygen-Limited Growth and Ethanolic Fermentation by Saccharomyces cerevisiae. Appl Biochem Biotechnol 84, 617–632. https://doi.org/10.1385/ABAB:84-86:1-9:617

Lee, S.C., Park, S., 2016. Removal of furan and phenolic compounds from simulated biomass hydrolysates by batch adsorption and continuous fixed-bed column adsorption methods. Bioresour Technol 216, 661–668. https://doi.org/10.1016/j.biortech.2016.06.007

Lin, R., Cheng, J., Ding, L., Song, W., Zhou, J., Cen, K., 2015. Inhibitory effects of furan derivatives and phenolic compounds on dark hydrogen fermentation. Bioresour Technol 196, 250–255. https://doi.org/10.1016/j.biortech.2015.07.097

Lopes, A., Azevedo-Silva, J., Carsanba, · Erdem, Pintado, M., Ana, ·, Oliveira, S., Ferreira, · Carlos, Pereira, J.O., Carvalho, A.P., Oliveira, C., 2023. Peptide extract from spent yeast improves resistance of Saccharomyces cerevisiae to oxidative stress. Applied Microbiology and Biotechnology 2023 1–13. https://doi.org/10.1007/S00253-023-12514-3

Lu, C., Dong, J., Yang, S.T., 2013. Butanol production from wood pulping hydrolysate in an integrated fermentation–gas stripping process. Bioresour Technol 143, 467–475. https://doi.org/10.1016/J.BIORTECH.2013.06.012

Marques, T.A., Neves, L.C.G., Rampazo, E.M., Deltrejo Junior, E.L., de Souza, F.C., Marques, P.A.A., 2015. <b>TRS value of sugarcane according to bioenergy and sugar levels. Acta Sci Agron 37, 347–353. https://doi.org/10.4025/actasciagron.v37i3.19065

McWilliam, I.G., Dewar, R.A., 1958. Flame Ionization Detector for Gas Chromatography. Nature 1958 181:4611 181, 760–760. https://doi.org/10.1038/181760a0

Merkl, R., Hrádková, I., Filip, V., Šmidrkal, J., 2010. Antimicrobial and antioxidant properties of phenolic acids alkyl esters. Czech Journal of Food Sciences 28 (2010), 275–279. https://doi.org/10.17221/132/2010-CJFS

Mushtaq, Z., Asghar, N., Waheed, M., 2019. COMPARATIVE EVALUATION OF DETOXIFICATION STRATEGIES FOR SUGARCANE BAGASSE HYDROLYSATE. J Anim Plant Sci 29, 1775–1783.

Nazir, A., Zahra, F., Sabri, M.U., Ghaffar, A., Ather, A.Q., Khan, M.I., Iqbal, M., 2021. Charcoal prepared from Bougainvillea spectabilis leaves as low cost adsorbent: Kinetic and equilibrium studies for removal of iron from aqueous solution. Zeitschrift fur Physikalische Chemie 235, 265–279. https://doi.org/10.1515/ZPCH-2019-1455/MACHINEREADABLECITATION/RIS

Nghiem, N.P., Montanti, J., Johnston, D.B., 2016. Sorghum as a renewable feedstock for production of fuels and industrial chemicals. AIMS Bioeng 3, 75–91. https://doi.org/10.3934/bioeng.2016.1.75

Pour, P.G., Takassi, M.A., Hamoule, T., 2014. Removal of Aluminum from water and industrial waste water. Oriental Journal of Chemistry 30, 1365–1369. https://doi.org/10.13005/OJC/300356

Preechakun, T., Pongchaiphol, S., Raita, M., Champreda, V., Laosiripojana, N., 2022. Detoxification of hemicellulose-enriched hydrolysate from sugarcane bagasse by activated carbon and macroporous adsorption resin. Biomass Convers Biorefin 1, 1–16. https://doi.org/10.1007/S13399-022-03596-6

Richard, D., Delgado Núñez, M. de L., Schweich, D., 2009. Adsorption of complex phenolic compounds on active charcoal: Adsorption capacity and isotherms. Chemical Engineering Journal 148, 1–7. https://doi.org/10.1016/j.cej.2008.07.023

Ronen, M., Shalaby, S., Horwitz, B.A., 2013. Role of the transcription factor ChAP1 in cytoplasmic redox homeostasis: imaging with a genetically encoded sensor in the maize pathogen Cochliobolus heterostrophus. Mol Plant Pathol 14, 786–790. https://doi.org/10.1111/MPP.12047

Shakoury-Elizeh, M., Protchenko, O., Berger, A., Cox, J., Gable, K., Dunn, T.M., Prinz, W.A., Bard, M., Philpott, C.C., 2010. Metabolic Response to Iron Deficiency in Saccharomyces cerevisiae. Journal of Biological Chemistry 285, 14823–14833. https://doi.org/10.1074/jbc.M109.091710

Souza, S.O., Costa, S.S.L., Brum, B.C.T., Santos, S.H., Garcia, C.A.B., Araujo, R.G.O., 2019. Determination of nutrients in sugarcane juice using slurry sampling and detection by ICP OES. Food Chem 273, 57–63. https://doi.org/10.1016/J.FOODCHEM.2018.03.060

Sperança, M.A., Nascimento, P.A.M., Pereira, F.M.V., 2021. Impurity in sugarcane juice as mineral content: A prospect for analysis using energy-dispersive X-ray fluorescence (EDXRF) and chemometrics. Microchemical Journal 164, 105951. https://doi.org/10.1016/J.MICROC.2021.105951

Suich, R., Derringer, G.C., 1980. Is the regression equation adequate?—a further note. Technometrics 22, 125–126. https://doi.org/10.1080/00401706.1980.10486110

Thai, C., Doherty, W., 2011. The composition of sugarcane juices derived from burnt cane and whole green cane crop. Proceedings of the 33rd Annual Conference of the Australian Society of Sugar Cane Technologists 2011.

Tungmunnithum, D., Drouet, S., Hano, C., 2022. Flavonoids from Sacred Lotus Stamen Extract Slows Chronological Aging in Yeast Model by Reducing Oxidative Stress and Maintaining Cellular Metabolism. Cells 11. https://doi.org/10.3390/CELLS11040599

Vicentini-Polette, C., Belé, J., Borges, M.T., Spoto, M., Verruma-Bernardi, M., 2019. Physicochemical and sensorial characterization of commercial sugarcane syrups [WWW Document]. Revista de Ciências Agrárias. https://doi.org/10.19084/rca.17279

Vohra, M., Manwar, J., Manmode, R., Padgilwar, S., Patil, S., 2014. Bioethanol production: Feedstock and current technologies. J Environ Chem Eng 2, 573–584. https://doi.org/10.1016/J.JECE.2013.10.013

Wang, X., Tsang, Y.F., Li, Y., Ma, X., Cui, S., Zhang, T.-A., Hu, J., Gao, M.-T., 2017. Inhibitory effects of phenolic compounds of rice straw formed by saccharification

during ethanol fermentation by Pichia stipitis. Bioresour Technol 244, 1059–1067. https://doi.org/10.1016/j.biortech.2017.08.096

Annex

Table 1. LC-ESI-UHR-QqTOF-MS results of phenolic compounds detected in sugarcane syrup with and without purification with activated charcoal. ND – Not detected. Different letters in the same row - (a) or (b) - represent the significant differences from a *t*-test (n = 3).

Proposed compound	Retention time (min)	Molecular Formula -H	*m/z* Measured $[M-H]^-$	MS/MS fragments	Error (ppm)	mSigma (Da)	Concentration (mg/L)	
							Non-purified sugarcane syrup	Purified sugarcane syrup
Hydroxybenzoic acids								
4-Hydroxybenzaldehyde	8.7	$C_7H_5O_2$	121.0295	121	2.7	3.4	2.8 ± 0.5	ND
o-Hydroxybenzoic acid	4.7	$C_7H_5O_3$	137.0244	108	1.3	5.4	1.5 ± 0.5	ND
4-Hydroxybenzoic acid	7.2	$C_7H_5O_3$	137.0221	93, 137	2.9	1.7	1.0 ± 0.1	ND
3,4-Dihydroxybenzaldehyde	6.9	$C_7H_5O_3$	137.0244	29, 72	0.4	9.6	0.9 ± 0.2	ND
Protocatechuic acid	5.7	$C_7H_5O_4$	153.0193	109, 153	3.2	5.4	1.3 ± 0.2	ND
2,6-Dihydroxybenzoate	8.5	$C_7H_5O_4$	153.0194	109	1.7	7.2	1.3 ± 1.1	ND
Gentisic acid	9.2	$C_7H_5O_4$	153.0193	65, 109	2.2	2.5	0.9 ± 1.0	ND
Hydroxybenzoic-4-β-glucoside	4.8	$C_{13}H_{15}O_8$	299.0717	108, 152	2.1	3.1	5.0 ± 1.0 (a)	1.1 ± 0.0 (b)
Gentisic acid derivatives	5.4	$C_{13}H_{15}O_9$	315.0722	108, 152	1.7	4.7	9.0 ± 0.8 (a)	1.2 ± 0.1 (b)
Hydroxycinnamic acids								
p-Coumaric acid	10.3	$C_9H_7O_3$	163.0401	119	1.9	8.0	5.4 ± 1.0	ND
p-Coumaric acid derivative	10.9	$C_9H_7O_3$	163.0401	119	1.9	8.0	0.3 ± 0.1	ND
Caffeic acid	8.5	$C_9H_8O_4$	179.0317	135, 179	3.0	6.3	0.9 ± 0.1	ND
Ferulic acid	9.7	$C_{10}H_9O_4$	193.0506	134	2.8	9.3	0.4 ± 0.1	ND
Isoferulic acid	11.2	$C_{10}H_9O_4$	193.0479	134, 161, 193	3.5	5.8	2.2 ± 0.1	ND
4-*p*-Coumaroylquinic acid	7.4	$C_{16}H_{17}O_8$	337.0929	119, 163	1.9	2.6	0.8 ± 0.2	ND
p-Coumaroylquinic acid	9.2	$C_{16}H_{17}O_8$	337.0929	93, 163, 173, 191	2.1	2.5	1.4 ± 0.3	ND
Neochlorogenic acid	6.3	$C_{16}H_{17}O_9$	353.0878	135, 179, 191	1.6	4.1	6.9 ± 1.3	ND
Chlorogenic acid	7.8	$C_{16}H_{17}O_9$	353.0878	191	1.7	3.2	5.9 ± 1.4	ND
4-Caffeoylquinic acid	8.0	$C_{16}H_{17}O_9$	353.0878	135, 173, 179, 191	2.2	2.0	3.8 ± 0.7	ND
5-*O*-Feruloylquinic acid	8.1	$C_{17}H_{19}O_9$	367.1035	134, 193	0.0	ND	3.7 ± 0.4	ND
trans-3-Feruloylquinic acid	9.8	$C_{17}H_{19}O_9$	367.0596	173	1.6	2.7	8.2 ± 0.8	ND
Caffeoylquinic acid	9.9	$C_{25}H_{24}O_{12}$	515.1195	515	3.2	7.3	5.2 ± 1.3	ND
Flavonoids								
tricin-7-*O*-glucoside	12.4	$C_{25}H_{31}O_{10}$	491.1826	329	4.2	13.5	0.5 ± 0.1	ND
Isoschaftoside	10.1	$C_{26}H_{27}O_{14}$	563.1406	353, 473	1.9	3.3	1.6 ± 0.5	ND
Apigenin 7-*O*-neohesperidoside	11.3	$C_{27}H_{29}O_{14}$	577.1563	293, 413	1.3	6.9	0.1 ± 0.1	ND
Tricin-*O*-neohesperoside isomer	13.4	$C_{29}H_{33}O_{16}$	637.1638	329	1.1	11.5	0.1 ± 0.1	ND
Tricin-7-*O*-rhamnosyl-glucuronide	13.1	$C_{29}H_{31}O_{17}$	651.1567	329	2.9	4.7	1.3 ± 0.4	ND
Tricin diglucuroniside	11.5	$C_{29}H_{29}O_{19}$	681.1322	351	1.0	15.7	0.8 ± 0.2	ND

Chapter 4

Production of β-farnesene from sugarbeet syrup by *Saccharomyces cerevisiae* in fed-batch fermentation

Luís Carlos Carvalho[a,b], Ana L. S. Oliveira[b], Erdem Carsanba[a,b], Ana Lopes[a,b], Tânia Leal[b], Mónica Ribeiro[a,b], Sara Fernandes[b], Manuela Pintado[b], Carla Oliveira[b]

[a] Amyris BioProducts Portugal, Unipessoal, Lda. Rua Diogo Botelho, 1327, 4169-005 Porto, Portugal

[b] CBQF - Centro de Biotecnologia e Química Fina - Laboratório Associado, Escola Superior de Biotecnologia, Universidade Católica Portuguesa, Rua Diogo Botelho, 1327, 4169-005 Porto, Portugal

Confidential

Abstract

Farnesene is currently produced in Brazil through industrial fermentation using sugarcane syrup as feedstock. However, its distribution from tropical regions is costly and originates large carbon footprints. Sugarbeet can be grown in milder climates and the use of beet syrup could allow local production of β-farnesene. Thus, this study aimed to evaluate the feasibility of utilizing sugarbeet syrup as an alternative feedstock in the β-farnesene fermentation process and to characterize its fermentation phenolic profile. For this, sugarbeet syrup composition was characterized, and a 2 L bioreactor fed-batch fermentation process was performed. Beet syrup presented high concentrations of glucose and hydroxymethylfurfural (HMF), at 193 and 1.4 g/L, respectively, and low concentrations of phenolic compounds (at 21.3 mg/L). Inoculum growth in bioreactor was optimized through fed-batch with initial 40 g/L of beet syrup TRS and subsequent 10 g/L pulses, and the desired cell density (wOD > 45) was obtained. Successful production fermentation was conducted for 8 days using beet syrup in fed-batch mode. Final β-farnesene concentration was 127 g/L, with 19.44 % and 1.70 g/L/h of cumulative β-farnesene yield and productivity, respectively. Consequently, beet syrup is herein demonstrated for the first time as a viable feedstock for β-farnesene production.

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

1. Introduction

Over the recent decades, fermentation has emerged as a prominent technique for biomanufacturing value-added compounds, leveraging renewable feedstocks (Amer and Baidoo, 2021; Boodhoo et al., 2022). For instance, sugarcane (*Saccharum officinarum* L.) and sugarbeet (*Beta vulgaris* L.) syrups have been supplied to *Saccharomyces cerevisiae* cultures to industrially produce molecules of interest, such as single cell protein, bioethanol, lactic acid and polylactic acid (Castro-Aguirre et al., 2016; Díaz et al., 2020; Kotzamanidis et al., 2002; Marzo et al., 2019; Muir and Anderson, 2022; Valladares-Diestra et al., 2022). Utilizing renewable cane and beet syrup in biomanufacturing offers a more sustainable alternative to the finite fossil resources traditionally employed in chemical synthesis (Meadows et al., 2016). In addition, utilizing these carbon sources requires fewer purification stages during production, making them not only more cost-effective but also environmentally friendly compared to pure sucrose (Abdel-Aleem, 2020).

Farnesene, a valuable sesquiterpene, is currently produced by Amyris in Brazil through the fermentation of cane syrup (Hill et al., 2020). The demand for farnesene, more specifically β-farnesene, has been increasing since this molecule has many applications in the cosmetic industry or as a surfactant, adjuvant, or a biofuel or biogas (Benjamin et al., 2016; Carsanba et al., 2021; Jacobs et al., 2017). For instance, β-farnesene can be converted to various oils, such as squalene (Brown et al., 2020; Hoff et al., 2021). This sustainable synthesis of squalene serves as an alternative to the traditional method that involves shark liver extraction (Mendes et al., 2022). Nevertheless, the distribution of farnesene-derived products from Brazil to other parts of the world poses challenges due to expensive shipping costs and significant carbon emissions, contributing to large carbon footprints (Chen et al., 2019). Furthermore, due to the crop's requirement for warm temperatures and ample water supply, the cultivation of sugarcane fields necessary for fermentation is limited to tropical regions (De Matos et al., 2020).

Incorporating additional feedstocks into the farnesene manufacturing process could expand the range of fermentation substrates, reducing reliance on cane syrup. This diversification offers more manufacturing options based on factors such as availability, cost, and regional demands (Hoff et al., 2021; Tropea, 2022). Diversification has the potential to enhance sustainability by significantly reducing supply chain demands in non-tropical regions through local farnesene production. Sugarbeet, hereby named as beet, is cultivated

in generally colder climates, such as Europe, North America, and Asia (Ritchie et al., 2023). Thus, utilizing beet syrup from these regions as feedstock could reduce the reliance on imports from Brazil and support regional self-sufficiency.

Feedstocks derived from beet, including juices, syrups or molasses, are not composed solely of pure sugars. Consequently, they contain additional molecules, including phenolic compounds, in their composition (Alexandri et al., 2019; Arjeh et al., 2022; Chen et al., 2017; Corleto et al., 2018). Phenolic compounds encompass a diverse range of molecules, and they can have effects on the fermentation process. These compounds possess antioxidant properties, which can contribute to an extended fermentation duration by promoting yeast health and longevity (Adeboye et al., 2014). Some works have reported the presence of antioxidant phenolic compounds in beet, such as phenolic acids, flavonoids, betaxanthins and betacyanins (Alexandri et al., 2019; Arjeh et al., 2022). On the other hand, phenolic compounds can have antimicrobial activity (Adeboye et al., 2014). Beet has been reported to have inhibitory compounds, such as ferulic acid (Chen et al., 2017).

To the best of our knowledge, beet syrup has primarily been utilized for bioethanol production through *S. cerevisiae* fermentation, and its industrial application in β-farnesene production has not been explored. Currently, only cane syrup has been utilized for this purpose. Therefore, it would be interesting to evaluate if beet syrup can also be employed in this process. Thus, this study aimed to assess the feasibility of employing beet syrup as a feedstock in the β-farnesene fermentation process. Additionally, it aimed to characterize the sugar and phenolic content of beet syrup and the fermentation broth during β-farnesene production. To achieve this, a 2 L bioreactor fed-batch fermentation process was conducted, where beet syrup was provided as the substrate for yeast fermentation.

2. Materials and Methods

2.1.Feedstock characterization

The beet syrup studied in this work was purchased from Grafschafter Krautfabrik (Meckenheim, Germany). The amounts of sugars, phenolic compounds, furfural, hydroxymethylfurfural (HMF), and acetic acid in the syrup as well as its antioxidant activity were assessed.

The sugars, HMF, furfural and acetic acid concentrations were measured through high performance liquid chromatography (HPLC) and a refractive index detector (RID), following the protocol NREL/TP-510-42618 (Sluiter et al., 2004). In an Aminex HPX-87H column (300 × 7.8 mm) with a pre-column (30 × 4.6 mm; Bio-rad, Hercules, USA), the chemicals were separated through a mobile phase with 5 mM sulfuric acid (Sigma-Aldrich, Missouri, USA) eluting at 0.6 mL/min and 50 °C. The RI detector (Agilent, Santa Clara, USA) was maintained at 35 °C. Individual calibration curves were constructed with standards of sucrose, glucose, fructose, HMF, furfural and acetic acid (Sigma-Aldrich, Missouri, USA).

The phenolic compounds were analyzed using LC-ESI-UHR-QqTOF-MS (liquid chromatography with ultrahigh-resolution, electrospray ionization, quadrupole time of flight, and mass spectrometry) following the methodology described by (Carvalho et al., 2022). The separation of compounds was achieved using a flow rate of 0.25 mL/min and an elution gradient between eluents A (0.1% aqueous formic acid) and B (acetonitrile with 0.1% formic acid). Calibration curves were constructed using standard concentrations of various phenolic compounds, including protocatechuic acid, vanillic acid, *p*-coumaric acid, caffeic acid, ferulic acid, chlorogenic acid, gentisic acid, 4-hydroxybenzaldehyde, 4-hydroxybenzoic acid, 3,4-dihydroxybenzaldehyde, syringic acid (Sigma-Aldrich), vitexin, diosmetin, naringenin, isoschaftoside, orientin, vitexin-2-O-rhamnoside (Extrasynthèse, France), tricin, naringenin-7-*O*-glucoside, and luteolin (Sigma-Aldrich). Duplicate samples were analyzed, and the results were reported as mg/L of syrup. The total phenolic content (TPC) was calculated as the sum of the concentrations of all identified phenolic compounds using LC-ESI-UHR-QqTOF-MS.

The antioxidant activity of the syrup was determined through the $ABTS^{\bullet+}$ (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)) and DPPH (2,2-diphenyl-1-picrylhydrazyl) methods (Oliveira et al., 2022; **Chapter 3**). Briefly, a solution of ABTS and potassium persulfate reacted with the sample and the inhibition of the formation of ABTS radicals was analyzed. Trolox was used as the reference compound. A microplate reader (BioTek Synergy H1, Agilent, Santa Clara, USA) was used to measure the absorbance at 734 nm. Multiple sample dilutions were used to determine the IC_{50} (sample concentration required to inhibit radical formation by 50 %) from the slope of the trendline between the ABTS inhibition and the sample equation.

Mineral content was determined through inductively coupled plasma optical emission spectrometry (ICP-OES; Oliveira et al., 2022), in an Optima 7000 DV ICP-OES (Perkin-Elmer, Massachusetts, USA). Samples were digested at a temperature reaching 190 ˚C and a pressure reaching 40 bar for 10 min in a microwave-assisted digester XPERT (BERGHOF, Eningen, Germany). Standard concentrations of potassium, calcium, magnesium, phosphorous, sodium, molybdenum, iron, aluminum, manganese and copper were used to construct the calibration curves. Samples were analyzed in triplicate and results presented as mg/L of syrup.

2.2.Microorganism and inoculum

An Amyris genetically engineered *S. cerevisiae* was used in this study, which produces β-farnesene constitutively. The yeast was activated from two 1 mL cryovials in 63 mL of culture medium and this culture was incubated in a 250 mL shake-flask during 42 h. Then, 5.25 mL of the first seed flask culture were transferred to a 1 L shake-flask with 144.75 mL of culture medium and the yeast was incubated for further 24 h. The same medium was used in both seed flasks, following the composition described in Carvalho et al., 2022. Both seed flasks were incubated at 30 °C at 200 rpm, in an incubator with a 5 cm orbital diameter (Eppendorf New Brunswick™ Innova® 44).

To simulate the complete industrial fermentation process of Amyris, two additional bioreactor steps were used to obtain the inoculum for the main fermentation. For this, 2.7 L working volume DASGIP® Eppendorf bioreactors were used. These seed cultures were performed according to what was described by Carvalho et al., 2022. Both cultures were maintained at a temperature of 30 °C, a pH of 5.0 and a minimal of 30 % dissolved oxygen. Oxygen was supplied through filtered air at a rate of 0.5 L/min for the first and 0.75 L/min for the second bioreactor seed culture. The first bioreactor step followed a batch mode, in which 70 g/L of total reducing sugars (TRS) were supplied to the cultures from the syrup. Two independent cultures were performed using beet syrup to supply the sugars. The second bioreactor step was operated as a batch and fed-batch culture, depending on the conditions tested. In batch mode, an initial quantity of syrup was provided to obtain 100 or 160 g/L of TRS concentration. In fed-batch mode, the cultures started with 20 or 40 g/L of TRS but were supplied with additional pulses of syrup to provide 10 or 20 g/L of TRS per pulse. The

timing of the syrup pulses was determined by a feedback-controlled pulse feeding algorithm. When sugars and ethanol reached low concentrations, the dissolved oxygen increased and triggered the addition of the syrup pulses.

2.3.Bioreactor fermentation

After the two seed shake-flask and bioreactor cultures, the yeast biomass was used to inoculate the main bioreactor fermentation. The fermentations were performed according to Carvalho et al., 2022. For this, 1.2 L of culture media was inoculated with 353 mL from the second seed bioreactor step. Two independent fed-batch fermentations were performed with beet syrup. The same feedback-controlled pulse algorithm was used to feed pulses of syrup to the cultures once sugars and ethanol were consumed. A draw-and-fill method was used to maintain at least 1.1 L of fermentation broth inside the bioreactors. In each day of fermentation, a volume of broth was removed, and a post-sterile addition (PSA) solution was added to the culture. Process conditions were maintained at an aeration of 1 L/min, a pH of 5.0 and a temperature of 30 °C.

2.4.Fermentation analysis

During the culture, the fermentation broth was analyzed to determine yeast cell density through washed optical density (wOD) measured at 600 nm in a Shimadzu UV-1900 UV-VIS spectrophotometer, according to Carvalho et al., 2022. Briefly, the fermentation broth was centrifuged at 12300 × *g* for 5 min, the supernatant was removed, and water was added to restore the volume. Dilutions were made to obtain absorbance between 0.1 to 0.6.

The supernatant of the fermentation broth was used to determine sugar consumption through HPLC-RID and according to the method described in section 2.1.

The concentration of β-farnesene in the fermentation broth was determined according to Carvalho et al., 2022, through gas chromatography (GC) with a flame ionization detector (FID). The β-farnesene extraction was done with a methanol solution. The fermentation broth was analyzed in duplicate. Three fermentation parameters of the yeast cultures were determined. Namely, yield of biomass to substrate ($Y_{X/S}$), yield of farnesene to substrate ($Y_{P/S}$) and farnesene productivity (P_P) were calculated as described in **Chapter 3**. During the

production fermentation, which lasted for 8 days, interval $Y_{P/S}$ and P_P were also measured, using the production of farnesene and consumption of TRS between each fermentation day.

2.5.Statistical analysis

The statistical analysis of the data was performed by the software STATISTICA, version 14. The Shapiro-Wilk test was done to assess the normality of the data distribution. For determination of statistical significance of a result, student's *t*-test and one-way analysis of variance (ANOVA) with Fisher LSD post hoc tests were used. Statistical significance was obtained when the *p*-value was lower than 0.05.

3. Results and discussion

3.1.Syrup characterization

The beet syrup was characterized regarding its content in sugars, phenolic compounds, minerals, furfural, HMF (hydroxymethylfurfural), acetic acid, and antioxidant activity. Thirteen distinct phenolic compounds were identified in the beet syrup (Table 11). The predominant compound was ferulic acid, at about 15 mg/L, representing 71 % of total phenolic content (TPC; Table 11). Ferulic acid has also previously been reported as the phenolic compound with the highest concentration in sugarbeet molasses and red beet flesh (Chen et al., 2017; Płatosz et al., 2020), which corroborates the findings in our work. This phenolic compound has antimicrobial activity, being able to inhibit the growth of *S. cerevisiae* (Adeboye et al., 2017, 2015). Hence, the presence of ferulic acid at its minimum inhibitory concentration (MIC) in the fermentation medium can impede yeast growth. Studies have reported the MIC range of ferulic acid in *S. cerevisiae* to be between 0.8 and 2 g/L (Barber et al., 2000; Gu et al., 2015; Kimani et al., 2021; Merkl et al., 2010; Shirai et al., 2017).

Table 11. Phenolic compounds identified in beet syrup through the LC-ESI-QqTOF-HRMS methodology in negative mode. Values represent the average and standard deviation. HBA – hydroxybenzoic acids; HCA – hydroxycinnamic acids; ND – Not detected.

Proposed compound	Retention time	Molecular Formula -H	*m/z* Measured	MS/MS fragments	Error (ppm)	mSigma (Da)	Concentration (mg/L)
HBA							
o-Hydroxybenzoic acid	4.7	$C_7H_5O_3$	137.0244	108	1.3	5.4	1.59 ± 0.21 (a)
Protocatechuic acid	5.7	$C_7H_5O_4$	153.0193	109, 153	3.2	5.4	0.25 ± 0.09 (b)
2,6-Dihydroxybenzoate	8.5	$C_7H_5O_4$	153.0194	109	1.7	7.2	0.80 ± 0.01
Gentisic acid	9.2	$C_7H_5O_4$	153.0193	65, 109	2.2	2.5	0.42 ± 0.00 (b)
Hydroxybenzoic-4-β-glucoside	4.8	$C_{13}H_{15}O_8$	299.0717	108, 152	2.1	3.1	0.34 ± 0.03 (b)
Gentisic acid derivatives	5.4	$C_{13}H_{15}O_9$	315.0722	108, 152	1.7	4.7	0.87 ± 0.14 (b)
HCA							
Ferulic acid	9.7	$C_{10}H_9O_4$	193.0506	134	2.8	9.3	15.00 ± 2.65 (a)
Isoferulic acid	11.2	$C_{10}H_9O_4$	193.0479	134, 161, 193	3.5	5.8	0.48 ± 0.48 (b)
5-*O*-Feruloylquinic acid	8.1	$C_{17}H_{19}O_9$	367.1035	134, 193	0.0	ND	0.03 ± 0.03 (b)
Flavonoids							
Tricin-7-*O*-glucoside	12.4	$C_{25}H_{31}O_{10}$	491.1826	329	4.2	13.5	0.04 ± 0.02 (b)
Isoschaftoside	10.1	$C_{26}H_{27}O_{14}$	563.1406	353, 473	1.9	3.3	0.69 ± 0.17 (b)
Tricin/ 3',5'-*O*-Dimethyltricetin	17.7	$C_{17}H_{13}O_7$	329.0667	299	2.2	1.2	0.53 ± 0.01 (a)
Diosmetin	17.7	$C_{16}H_{12}O_6$	299.0502	284, 299	2.3	12.9	0.24 ± 0.16

Several studies have documented the presence of gallic acid, catechol, and epicatechin in sugarbeet (Alexandri et al., 2019; Arjeh et al., 2022). Furthermore, red beet has been found to contain betaxanthins, including vaulgaxanthin I and II, as well as betacyanins such as betanin, betanidin, isobetanin, and neobetanin (Corleto et al., 2018; Slavov et al., 2013). However, our study did not identify any of these compounds in beet syrup. It is plausible that higher temperatures were employed during the syrup manufacturing process, specifically during juice concentration through evaporation, which could have led to the degradation of various phenolic compounds (Alnaizy and Akgerman, 2000; M'hiri et al., 2014).

A prior study by Carvalho et al., 2022 investigated the phenolic content of cane syrup, which serves as the current feedstock for β-farnesene manufacturing. However, it is important to note that this study identified a distinct phenolic profile, encompassing a total of 39 phenolic compounds. In contrast to beet syrup analyzed in our work, the most predominant compound in cane syrup represented only 13 % of TPC, namely *trans*-3-feruloylquinic acid, at about 7 mg/L (Carvalho et al., 2022). In cane syrup, there were molecules included that were not identified in beet syrup, such as 4-hydroxybenzaldehyde and 4-hydroxybenzoic acid from hydroxybenzoic acids (HBA), *p*-coumaric acid and *trans*-3-feruloylquinic acid from hydroxycinnamic acids (HCA), and tricin diglucuroniside and

neoschaftoside from flavonoids (Carvalho et al., 2022). Therefore, beet syrup contained 3 times fewer phenolic compounds than cane syrup. Compounds in common between both syrups include protocatechuic acid, gentisic acid, ferulic acid, isoschaftoside and 3',5'-*O*-dimethyltricetin.

The TPC of beet syrup was determined to be 21.27 mg/L (Table 12). This concentration of phenolic compounds aligns with the studies conducted by Valli et al., 2012 and Grabek-Lejko and Tomczyk-Ulanowska, 2013, which investigated the TPC difference between cane and beet molasses. Both studies consistently reported a significant decrease in phenolic compound concentrations in beet molasses, ranging from 6 to 10 times lower compared to cane molasses. Our results are consistent with these findings, confirming the comparatively lower TPC in beet syrup.

Table 12. Characterization of beet syrup used in the fermentations regarding contents in phenolic, sugar and mineral contents, as well as antioxidant activity. The values represent the average and standard deviation. HBA – hydroxybenzoic acids; HCA – hydroxycinnamic acids; TPC – total phenolic content; ND – Not detected.

Parameter group	Parameter	Concentration
Phenolic content (mg/L)	HBA	4.27 ± 0.21
	HCA	15.50 ± 3.16
	Flavonoids	1.49 ± 0.37
	TPC	21.27 ± 3.73
Antioxidant activity (g syrup/L)	ABTS IC_{50}	27.36 ± 3.88
	DPPH IC_{50}	14.76 ± 0.28
Sugar content (g/L)	Sucrose	297.91 ± 6.28
	Fructose	197.36 ± 0.35
	Glucose	192.94 ± 0.13
	TRS	703.89 ± 6.12
Mineral content (mg/L)	Potassium	5020.88 ± 80.73
	Calcium	201.97 ± 8.53
	Magnesium	739.13 ± 26.83
	Phosphorous	737.87 ± 34.44
	Sodium	292.24 ± 3.88
	Molybdenum	ND
	Iron	ND
	Aluminum	59.81 ± 0.87
	Manganese	27.84 ± 0.74
	Copper	1.06 ± 0.10
Other compounds (g/L)	HMF	1.40 ± 0.35
	Furfural	0.17 ± 0.01
	Acetic acid	0.43 ± 0.06

The phenolic class with highest concentration was HCA, representing 73 % of TPC. This was followed by HBA at 20 % and flavonoids at 7 %. Sugarcane syrup has 2.4-fold the TPC obtained in the beet syrup of the current work (Carvalho et al., 2022). The lower phenolic content in beet syrup was found in all three classes of phenolic compounds, namely HBA, HCA and flavonoids. The biggest difference between the beet and cane syrups was observed in the flavonoid class (at 8.3-fold lower in beet). Thus, the lower phenolic content observed in beet syrup may be responsible for providing both lower antioxidant and antimicrobial activities.

Understanding the effects of phenolic compounds on yeast fermentation is essential for the development of strategies to control and modulate their impact. Phenolic compounds can play a crucial role in yeast fermentation, as they can have both positive and negative effects on yeast performance depending on their concentration. At lower concentrations, phenolic compounds can act as antioxidants, protecting yeast cells from oxidative stress and promoting cell viability and growth. However, higher concentrations of phenolic compounds can have inhibitory effects on yeast performance, affecting yeast growth, metabolism, and fermentation kinetics. They can interfere with cell membrane integrity, inhibit enzyme activity, and disrupt cellular processes. Nonetheless, the impact of phenolic compounds on yeast performance is dose-dependent: moderate concentrations may exert positive effects, while excessive amounts can have detrimental effects. A previous work by our group (**Chapter 3**) reports the study of the effect of phenolic compounds from sugarcane syrup in batch fermentation. The β-farnesene production by *S. cerevisiae* in the presence of 7.1 mg/L and 0.3 mg/L of TPC was compared, and it was found that, in bioreactor, the reduction in phenolic content increased the biomass yield by 3 % and biomass production by 12 %, while no impact on farnesene productivity was observed. In the present work, the concentration of phenolic compounds found in the beet syrup may also be enough to influence the growth and metabolism of the yeast.

The antioxidant activity of syrup was measured through IC_{50}: the concentration of syrup required to inhibit the formation of 50 % of free radicals. Beet syrup IC_{50} for ABTS was almost 2-fold the IC_{50} for DPPH (Table 12). This suggests that beet syrup contains a higher concentration of liposoluble molecules that exhibit antioxidant activity (Koss-Mikołajczyk et al., 2019). Overall, considering the different solvent systems used in ABTS (water) and DPPH (methanol) assays, lipophilic compounds may exhibit different behaviors

and potentially show better performance in the DPPH assay due to the more lipophilic nature of methanol. The antioxidant capacity of the feedstock can play a crucial role in reducing oxidative stress in yeast and prolonging fermentations (Munteanu and Apetrei, 2021). According to the study by Valli et al., 2012 , the antioxidant activity of beet molasses was assessed, and its DPPH IC_{50} value was determined to be 7.25 g/L. In our study, the DPPH IC_{50} value of beet syrup was found to be 14.76 g/L, as shown in Table 12. These findings suggest that beet molasses may contain a greater concentration of antioxidant compounds compared to beet syrup. Indeed, the difference in antioxidant capacity between beet molasses and beet syrup can be attributed to the concentration of phenolic compounds. During the production of molasses, the sugars are removed, leading to a higher concentration of antioxidant phenolic compounds. This concentration process enhances the antioxidant activity of molasses compared to the original syrup. Additionally, the study by Valli et al., 2012 compared the antioxidant capacity of cane and found that cane molasses exhibited 70 % higher antioxidant activity. This further supports the concept that molasses, regardless of the source, can have higher antioxidant capacity compared to syrups. Furthermore, in the current work the TPC of beet syrup was lower than that of cane syrup (from Carvalho et al., 2022), which aligns with the observed decreased antioxidant activity in beet syrup. Taken together, these findings indicate that the concentration of phenolic compounds, as reflected by the TPC, plays a significant role in the antioxidant capacity of beet syrup and molasses. Other non-phenolic antioxidant compounds have also been described in beetroot. For instance, ascorbic acid, betalain and carotenoids, such as β-carotene and lutein have been identified (Lechner and Stoner, 2019; Masih et al., 2019). However, in our work none of these compounds were identified.

Total sugar concentration in beet syrup was 703.89 g/L, with sucrose as the main sugar, representing 42 % of TRS (Table 12). Other works have reported the concentration of sucrose in beet syrup at 45 or 58 % of dry weight, which is 2 to 3-fold the 22 % of dry weight obtained in our work (Bahrami et al., 2020; Tan et al., 2015; Vučurović et al., 2019). Furthermore, beet syrup contained significant concentrations of fructose and glucose, both reaching nearly 200 g/L. These elevated levels suggest that the sucrose present in the beet plant underwent conversion into its monomers during the syrup manufacturing process. This conversion is likely attributed to high temperatures employed during beet juice evaporation (Clarke et al., 1997). Consequently, the beet syrup brought a substantial concentration of

monomers to fermentation. However, it is worth noting that high glucose levels can be suboptimal for *S. cerevisiae* fermentation due to the Crabtree effect, which can hinder yeast growth (Merico et al., 2007).

Minerals are essential for the growth, metabolism, and overall performance of *S. cerevisiae* during fermentation. This is the case of potassium, magnesium, calcium, manganese, iron, zinc and copper. Their presence in the fermentation broth ensures optimal yeast activity, higher fermentation efficiency, and the production of desirable fermentation products (de Souza et al., 2015). However, extreme concentrations of certain minerals, such as potassium and calcium, may cause osmotic stress and hinder the fermentation (Amorin et al., 2009). In beet syrup, results showed potassium as the most abundant mineral, with a concentration of approximately 5000 mg/L (Table 12). Then, magnesium and phosphorus were present at approximately 700 mg/L each. Gencturk and Ulgen, 2022 have analyzed the mineral content of beet syrup and found a similar concentration of potassium (around 5400 mg/L). The study found magnesium levels in beet syrup to be around seven times lower and calcium levels approximately eight times higher compared to our results. Previous research suggests that potassium and calcium concentrations in beet molasses are approximately ten times higher than in beet syrup, while sodium levels are detected at a thirty-fold higher concentration (Filipčev et al., 2010). This discrepancy is expected due to the nature of molasses, which are byproducts resulting from the removal of sugars and tend to concentrate other compounds present in the beet syrup.

In addition, the mineral profile of beet syrup was found to be different from what was found previously in cane syrup (**Chapter 3**). Thus, the nutrients – besides the sugars - that each syrup would supply to the yeast may also be different, which might influence the fermentation in different ways. In **Chapter 3**, cane syrup was found to contain 6200 mg/L of potassium. And, phosphorous, sodium, manganese and copper were found to be present in beet syrup at higher concentrations than cane syrup, at 7.6, 4.4, 3.6 and 2.0-fold, respectively. On the other hand, our work also presented beet syrup with decreased concentrations of calcium by 85 %. Regarding the macronutrients, the decreased levels of phosphorous may have an influence on the fermentation process, since it is an essential nutrient to the fermentation (de Souza et al., 2015). However, it worth noting that this nutrient is supplied to the fermentation in the form of ammonium phosphate monobasic.

Among the other compounds analyzed in beet syrup, hydroxymethylfurfural (HMF) was present at the highest concentration, 1.4 g/L (Table 12). HMF generation during the substrate manufacturing process has been associated with the use of elevated temperatures - at least 160 ˚C (Cheng et al., 2020). These results also suggest that the beet syrup was manufactured at high temperatures. The presence of HMF has also been linked with yeast growth inhibition and reduction in product formation (Gencturk and Ulgen, 2022). Thus, the high concentration of HMF can potentially hinder the overall yeast fermentation process.

3.2.Optimization of the second seed step

The objective of the second bioreactor seed step was to obtain a culture with a final wOD of at least 45 to inoculate the main fermenter. However, slow yeast growth was found using beet syrup in batch mode. An initial TRS of 100 g/L supplied from beet syrup in batch mode resulted in a final wOD of only 15.2 (Table 13). Increasing the initial TRS to 160 g/L also led to insufficient growth, with a final wOD of 7.7. In fact, the growth was inferior when supplying more sugars in the beginning of the batch culture. This indicated that the cultures were being inhibited by any component, or components, present in the syrup, since the increase of its concentration resulted in decreased yeast growth. The two main molecules present in the culture medium that could inhibit the yeast growth were glucose and HMF. In fact, in the beginning of the culture with 160 g/L of TRS, glucose was present at 42.6 g/L (data not shown), which is enough to slow yeast growth due to the Crabtree effect (Gibart et al., 2021; Merico et al., 2007). The concentration of HMF in the fermentation process was 0.28 g/L, which poses a potential threat to the growth of *S. cerevisiae*. Research conducted by Rafiqul et al., 2015 have found that HMF exhibits inhibitory effects on yeast growth and bioethanol production, at concentrations as low as 0.1 g/L. This inhibition occurs through the reduction of sugar intake and protein synthesis, further underscoring the adverse impact of HMF on yeast metabolism during fermentation (Banu Jamaldheen et al., 2022).

Table 13. Fermentation parameters after 42 h of *S. cerevisiae* culture in the second seed bioreactor step using beet syrup as carbon source. The values represent the average and standard deviation. wOD – washed optical density; $Y_{X/S}$ – yield of biomass to substrate.

Condition	Final wOD	Sugars consumed (g/L)	$Y_{X/S}$ (g/g)
Batch - 100 g/L TRS	15.2 ± 0.5 (d)	31.2 ± 1.1 (g)	0.487 ± 0.016 (b)
Batch- 160 g/L TRS	7.7 ± 0.3 (e)	40.6 ± 0.5 (f)	0.189 ± 0.008 (e)
Fed-batch - 20 g/L initial TRS + 10 g/L pulses	45.3 ± 0.1 (c)	93.7 ± 0.3 (e)	0.483 ± 0.001 (bc)
Fed-batch - 20 g/L initial TRS + 20 g/L pulses	47.5 ± 1.3 (c)	116.5 ± 0.2 (c)	0.408 ± 0.011 (d)
Fed-batch - 40 g/L initial TRS + 10 g/L pulses	59.1 ± 0.7 (b)	132.8 ± 0.0 (b)	0.445 ± 0.005 (bd)
Fed-batch - 40 g/L initial TRS + 20 g/L pulses	45.9 ± 0.7 (c)	92.4 ± 0.1 (e)	0.497 ± 0.008 (b)

Therefore, optimization of the second bioreactor seed step was performed by applying fed-batch mode to lower the initial concentration of glucose and HMF, and increase the final biomass (Mesquita et al., 2019). For this, two initial TRS concentrations were tested, 20 and 40 g/L, with two pulse concentrations of TRS addition during the culture, 10 and 20 g/L (Table 13). These pulses were supplied when the sugars were finishing, and dissolved oxygen increased. Initial glucose concentration in these fed-batch cultures was 6.6 and 12.5 g/L, respectively, depending on the syrup concentration (data not shown). Initial HMF concentration was up to 0.04 g/L (data not shown). These lower concentrations of glucose and HMF can enhance this biomass production step due to the reduction of their inhibitory effects. Results showed that the cultures supplied with 20 g/L of initial TRS from beet syrup started feeding syrup 5 to 7 h earlier than the ones supplied with 40 g/L (Figure 17). The total volume of syrup supplied to the bioreactors was the highest for the culture with 20 g/L of initial TRS and 10 g/L pulses. All the fed-batch conditions tested improved the growth of the yeast and increased the final wOD to at least 45 (Table 13). The culture with the highest final biomass was supplied with 40 g/L of initial TRS and pulses containing 10 g/L of TRS (Table 13). The culture from this condition reached a final wOD of 59.1 and was selected to inoculate the main production fermenter.

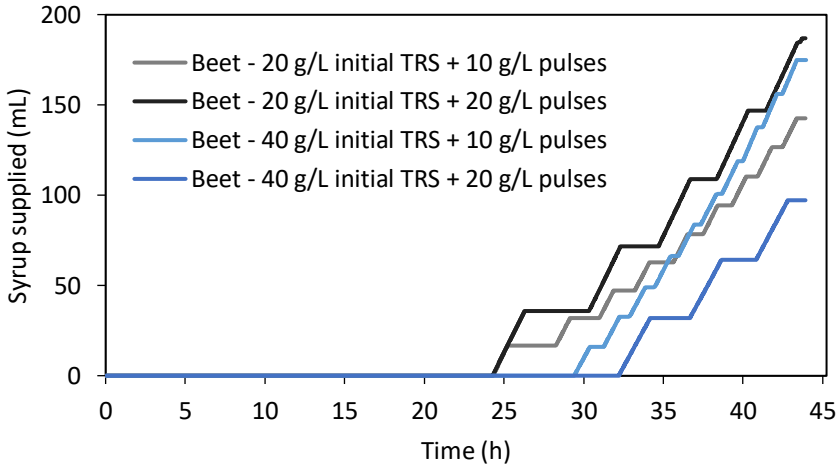

Figure 17. Volume of beet syrup supplied to the second seed bioreactor step in fed-batch mode of *S. cerevisiae* culture. TRS – total reducing sugars.

3.3.Main fermentation

During the main fermentation, the cultures fed with beet syrup reached a high cell density, presenting a wOD of around 100 to 120 after the second day of fermentation (Figure 18A). Production of β-farnesene increased over time until the last day of fermentation (Figure 18A). The cultures reached a final β-farnesene concentration of 127 g/L. Sugar consumption and farnesene accumulation (Figure 18B) also increased steadily over the fermentation time, reaching a total of 2114 and 411 g, respectively, at the end of the fermentation.

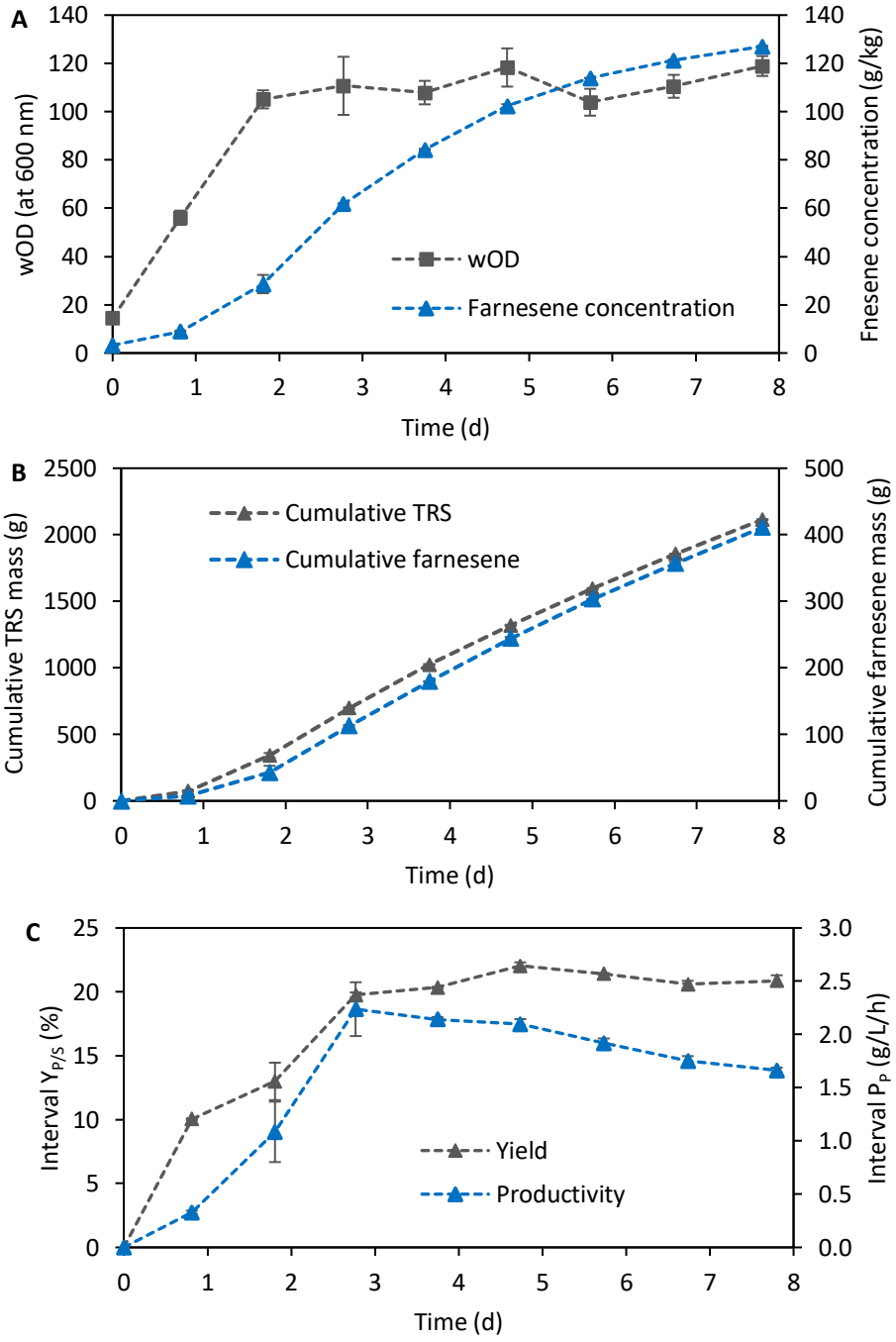

Figure 18. Main fed-batch fermentation of *S. cerevisiae* for β-farnesene production using beet syrup. A: Washed optical density (wOD; measured at 600 nm) and farnesene concentration; B: Cumulative mass of TRS and farnesene; C: interval $Y_{P/S}$ (yield of β-farnesene to substrate) and P_P (β-farnesene productivity). The values represent the average and standard deviation.

The work by Meadows et al., 2016, which employed a *S. cerevisiae* fermentation process using sugarcane as feedstock, obtained a final concentration of β-farnesene at around 130 g/L. Production of β-farnesene has also been performed using other microorganisms, however, with lower concentration results. For instance, the highest β-farnesene

concentration was obtained in 2 L fermenters in fed-batch mode with *Yarrowia lipolytica* at 22.8 g/L using pure glucose (Shi et al., 2021) and 7.38 g/L using lignocellulosic corn stover (Bi et al., 2022). In *Escherichia coli*, the highest concentration obtained was 10.31 g/L from lignocellulosic corncob in 5 L bioreactors operated in batch mode (Yao et al., 2020). The concentration of β-farnesene obtained in the present work reached the same level as the maximum produced from sugarcane syrup, highlighting the potential of using beet syrup as alternative feedstock.

The interval farnesene yield was stable after the third day of fermentation, reaching a maximum of 22 % at day 5 (Figure 18C). The interval farnesene productivity also increased until day 3. However, after this day, the interval productivity presented a decline over time from a maximum of 2.25 g/L/h to 1.66 g/L/h.

The final cumulative farnesene yield and productivity of the fermentations using beet syrup were 19.44 % and 1.70 g/L/h, respectively (data not shown). In the work by Meadows et al., 2016, the farnesene yield from sugarcane syrup was 17.3 %, while the productivity was 2.24 g/L/h. Based on these results, it can be concluded that the yeast achieved a favorable conversion yield from beet sugars to farnesene, as indicated by the higher farnesene yield, as compared to cane sugars yield. However, a comparison between productivities implies that the consumption and conversion rate of sugar were lower in our fermentations with beet syrup, as compared to those with sugarcane syrup.

The production of lactic acid in batch bioreactor fermentation, supplied with beet or cane syrup, was compared (Calabia and Tokiwa, 2007). It was found a decreased growth with beet syrup, with the generation of only one third of biomass. Lactic acid yield was substantially lower (by 7 %) when using beet syrup. The authors attributed the differences in growth and production to the decreased nutrients and to the presence of inhibitory compounds found in beet syrup, such as organic acids and aldehydes, phenolic compounds, and heavy metals.

In terms of phenolic content during the main fermentation (Figure 19), an initial increase in TPC was observed in the first three days, followed by phenolic concentrations ranging from 5.9 to 11.6 mg/L throughout the remaining fermentation time. Throughout the stationary phase, the average TPC remained at 8.6 mg/L, as depicted in Figure 19. During the fed-batch fermentation, a total of 3 L of beet syrup were supplied, with syrup being 77

% of the entire volume of the fermentation broth (initial culture medium + syrup + base). Due to this supply of large quantities of syrup during the fermentation, it was expected that the phenolic content in the syrup would be transferred to the fermentation broth. However, the TPC of the fermentation broth was only 41 % of the phenolic concentration found in the beet syrup. This difference in TPC between the fermentation broth and the syrup may have also occurred due to the metabolization of multiple phenolic compounds (Adeboye et al., 2015). The yeast *S. cerevisiae* is inhibited by certain phenolic compounds, and it can catabolize and metabolize these molecules. For example, the fermentation broth contained ferulic acid at a concentration of 4.0 mg/L, representing only 27% of the concentration found in the beet syrup. Similar differences occurred regarding the concentrations of isoferulic acid (at 13 %), diosmetin (at 35 %), tricin (at 25 %), 5-*O*-feruloylquinic acid (at 36 %) and isoschaftoside (at 31 %). Degradation of ferulic acid by *S. cerevisiae* has been previously reported by Adeboye et al., 2015; Carvalho et al., 2022; Fletcher et al., 2019; Oliveira et al., 2023. The yeast's ability to convert certain phenolic compounds present in the fermentation broth is further supported by the elevated concentration of protocatechuic acid observed in the fermentation broth (data not shown). This concentration was found to be 2.4 times higher than that found in the beet syrup. This finding aligns with the research conducted by Oliveira et al., 2023, who also reported an increase in the concentration of protocatechuic acid after *S. cerevisiae* fermentation of sugarcane straw. In our work, the concentration of tricin-7-*O*-glucoside was also 2.9-fold the concentration found in the beet syrup. However, this compound has previously been shown to decrease after yeast fermentation (Oliveira et al., 2023).

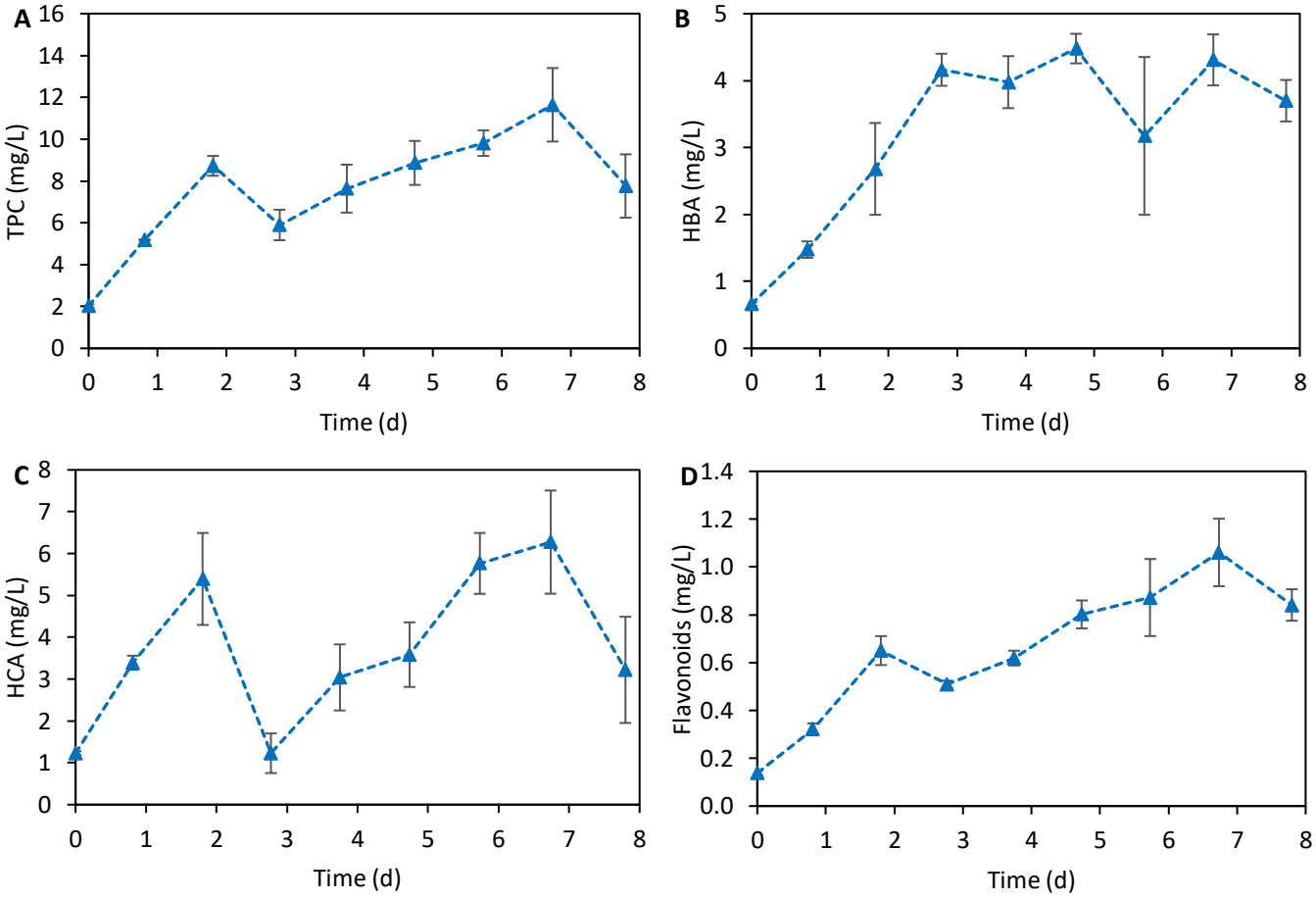

Figure 19. Phenolic content measured during the main fed-batch fermentations of *S. cerevisiae* for β-farnesene production using beet syrup. A: total phenolic content - TPC; B: hydroxybenzoic acids - HBA; C: hydroxycinnamic acids – HCA; D: flavonoids. The values represent the average and standard deviation.

4. Conclusions

Characterization of beet syrup revealed high concentrations of possible inhibitors, such as glucose at 193 g/L and HMF at 1.4 g/L, and low concentration of phenolic compounds at 21.3 mg/L. Growth of inoculum in bioreactor was optimized to obtain the target cell density (wOD = 45) using initial 40 g/L of beet syrup TRS and fed-batch with 10 g/L TRS pulses. The production fermentation was successfully performed with beet syrup, and the production of β-farnesene was accomplished for the first time with this feedstock. A total of 411 g of β-farnesene was produced from 2114 g of beet syrup TRS in 8 days fed-batch fermentation. Final β-farnesene parameters demonstrate the ability of employing beet syrup as a feedstock and show the potential for β-farnesene biosynthesis utilizing this alternative carbon source. Improvement of the fermentation process with beet syrup may be

conducted by using a syrup resulting from a manufacturing process that does not produce high degradation of the sucrose molecule into its monomers or the generation of HMF. Given that sugarcane syrup is the primary feedstock used for farnesene fermentation, it would be valuable to compare the production of β-farnesene from beet syrup and cane syrup in parallel fermentations. This work opens perspectives for developing beet syrup as an alternative feedstock to produce β-farnesene.

References

Abdel-Aleem, W.M., 2020. Effect of Sugarcane Juice Pre-Treatment on the Quality and Crystallization of Sugarcane Syrup (Treacle). J Food Process Technol 11, 1–10. https://doi.org/10.35248/2157-7110.20.11.834

Adeboye, P.T., Bettiga, M., Aldaeus, F., Larsson, P.T., Olsson, L., 2015. Catabolism of coniferyl aldehyde, ferulic acid and p-coumaric acid by Saccharomyces cerevisiae yields less toxic products. Microb Cell Fact 14, 149. https://doi.org/10.1186/s12934-015-0338-x

Adeboye, P.T., Bettiga, M., Olsson, L., 2017. ALD5, PAD1, ATF1 and ATF2 facilitate the catabolism of coniferyl aldehyde, ferulic acid and p-coumaric acid in Saccharomyces cerevisiae. Sci Rep 7, 42635. https://doi.org/10.1038/srep42635

Adeboye, P.T., Bettiga, M., Olsson, L., 2014. The chemical nature of phenolic compounds determines their toxicity and induces distinct physiological responses in Saccharomyces cerevisiae in lignocellulose hydrolysates. AMB Express 4, 46. https://doi.org/10.1186/s13568-014-0046-7

Alexandri, M., Schneider, R., Papapostolou, H., Ladakis, D., Koutinas, A., Venus, J., 2019. Restructuring the Conventional Sugar Beet Industry into a Novel Biorefinery: Fractionation and Bioconversion of Sugar Beet Pulp into Succinic Acid and Value-Added Coproducts. ACS Sustain Chem Eng 7, 6569–6579. https://doi.org/10.1021/ACSSUSCHEMENG.8B04874

Alnaizy, R., Akgerman, A., 2000. Advanced oxidation of phenolic compounds. Advances in Environmental Research 4, 233–244. https://doi.org/10.1016/S1093-0191(00)00024-1

Arjeh, E., Khodaei, S.M., Barzegar, M., Pirsa, S., Karimi Sani, I., Rahati, S., Mohammadi, F., 2022. Phenolic compounds of sugar beet (Beta vulgaris L.): Separation method, chemical characterization, and biological properties. Food Sci Nutr 10, 4238–4246. https://doi.org/10.1002/FSN3.3017

Bahrami, M.E., Honarvar, M., Ansari, K., Jamshidi, B., 2020. Measurement of quality parameters of sugar beet juices using near-infrared spectroscopy and chemometrics. J Food Eng 271, 109775. https://doi.org/10.1016/J.JFOODENG.2019.109775

Banu Jamaldheen, S., Kurade, M.B., Basak, B., Yoo, C.G., Oh, K.K., Jeon, B.H., Kim, T.H., 2022. A review on physico-chemical delignification as a pretreatment of lignocellulosic biomass for enhanced bioconversion. Bioresour Technol 346, 126591. https://doi.org/10.1016/J.BIORTECH.2021.126591

Barber, M.S., McConnell, V.S., Decaux, B.S., 2000. Antimicrobial intermediates of the general phenylpropanoid and lignin specific pathways. Phytochemistry 54, 53–56. https://doi.org/10.1016/S0031-9422(00)00038-8

Benjamin, K.R., Silva, I.R., Cherubim, J.P., McPhee, D., Paddon, C.J., 2016. Developing Commercial Production of Semi-Synthetic Artemisinin, and of β-Farnesene, an Isoprenoid Produced by Fermentation of Brazilian Sugar. J Braz Chem Soc 27, 1339–1345. https://doi.org/10.5935/0103-5053.20160119

Bi, H., Xv, C., Su, C., Feng, P., Zhang, C., Wang, M., Fang, Y., Tan, T., 2022. β-Farnesene Production from Low-Cost Glucose in Lignocellulosic Hydrolysate by Engineered Yarrowia lipolytica. Fermentation 8, 532. https://doi.org/10.3390/FERMENTATION8100532/S1

Brown, J., Hahn, H., Rice, L., Vettel, P., Wells, J., 2020. Farnesene-Derived Polyolefin Base Oils. Synthetics, Mineral Oils, and Bio-Based Lubricants 449–472. https://doi.org/10.1201/9781315158150-26

Calabia, B.P., Tokiwa, Y., 2007. Production of D-lactic acid from sugarcane molasses, sugarcane juice and sugar beet juice by Lactobacillus delbrueckii. Biotechnol Lett 29, 1329–1332. https://doi.org/10.1007/S10529-007-9408-4

Carsanba, E., Pintado, M., Oliveira, C., 2021. Fermentation Strategies for Production of Pharmaceutical Terpenoids in Engineered Yeast. Pharmaceuticals 14. https://doi.org/10.3390/PH14040295

Carvalho, L.C., Oliveira, A.L.S., Carsanba, E., Pintado, M., Oliveira, C., 2022. Phenolic compounds modulation in β-farnesene fed-batch fermentation using sugarcane syrup as feedstock. Ind Crops Prod 188, 115721. https://doi.org/10.1016/J.INDCROP.2022.115721

Castro-Aguirre, E., Iñiguez-Franco, F., Samsudin, H., Fang, X., Auras, R., 2016. Poly(lactic acid)—Mass production, processing, industrial applications, and end of life. Adv Drug Deliv Rev 107, 333–366. https://doi.org/10.1016/J.ADDR.2016.03.010

Chen, J., Fei, Y., Wan, Z., 2019. The relationship between the development of global maritime fleets and GHG emission from shipping. J Environ Manage 242, 31–39. https://doi.org/10.1016/J.JENVMAN.2019.03.136

Chen, M., Zhao, Z., Meng, H., Yu, S., 2017. The antibiotic activity and mechanisms of sugar beet (Beta vulgaris) molasses polyphenols against selected food-borne pathogens. LWT - Food Science and Technology 82, 354–360. https://doi.org/10.1016/J.LWT.2017.04.063

Cheng, M.H., Sun, L., Jin, Y.S., Dien, B., Singh, V., 2020. Production of xylose enriched hydrolysate from bioenergy sorghum and its conversion to β-carotene using an engineered Saccharomyces cerevisiae. Bioresour Technol 308, 123275. https://doi.org/10.1016/J.BIORTECH.2020.123275

Clarke, M.A., Edye, L.A., Eggleston, G., 1997. Sucrose Decomposition in Aqueous Solution, and Losses in Sugar Manufacture and Refining. Adv Carbohydr Chem Biochem 52, 441–470. https://doi.org/10.1016/S0065-2318(08)60095-5

Corleto, K.A., Singh, J., Jayaprakasha, G.K., Patil, B.S., 2018. Storage Stability of Dietary Nitrate and Phenolic Compounds in Beetroot (Beta vulgaris) and Arugula (Eruca sativa) Juices. J Food Sci 83, 1237–1248. https://doi.org/10.1111/1750-3841.14129

De Matos, M., Santos, F., Eichler, P., 2020. Sugarcane world scenario. Sugarcane Biorefinery, Technology and Perspectives 1–19. https://doi.org/10.1016/B978-0-12-814236-3.00001-9

de Souza, R.B., de Menezes, J.A.S., de Souza, R. de F.R., Dutra, E.D., de Morais, M.A., 2015. Mineral Composition of the Sugarcane Juice and Its Influence on the Ethanol Fermentation. Appl Biochem Biotechnol 175, 209–222. https://doi.org/10.1007/S12010-014-1258-7

Díaz, A.B., González, C., Marzo, C., Caro, I., Blandino, A., 2020. Feasibility of exhausted sugar beet pulp as raw material for lactic acid production. J Sci Food Agric 100, 3036–3045. https://doi.org/10.1002/JSFA.10334

Filipčev, B., Lević, L., Bodroža-Solarov, M., Mišljenovic, N., Koprivica, G., 2010. Quality Characteristics and Antioxidant Properties of Breads Supplemented with Sugar Beet Molasses-Based Ingredients. 13, 1035–1053. https://doi.org/10.1080/10942910902950526

Fletcher, E., Gao, K., Mercurio, K., Ali, M., Baetz, K., 2019. Yeast chemogenomic screen identifies distinct metabolic pathways required to tolerate exposure to phenolic fermentation inhibitors ferulic acid, 4-hydroxybenzoic acid and coniferyl aldehyde. Metab Eng 52, 98–109. https://doi.org/10.1016/j.ymben.2018.11.010

Gencturk, E., Ulgen, K.O., 2022. Understanding HMF inhibition on yeast growth coupled with ethanol production for the improvement of bio-based industrial processes. Process Biochemistry 121, 425–438. https://doi.org/10.1016/J.PROCBIO.2022.07.015

Gibart, L., Khoodeeram, R., Bernot, G., Comet, J.P., Trosset, J.Y., 2021. Regulation of Eukaryote Metabolism: An Abstract Model Explaining the Warburg/Crabtree Effect. Processes 2021, Vol. 9, Page 1496 9, 1496. https://doi.org/10.3390/PR9091496

Grabek-Lejko, D., Tomczyk-Ulanowska, K., 2013. Phenolic content, antioxidant and antibacterial activity of selected natural sweeteners available on the Polish market. J Environ Sci Health B 48, 1089–1096. https://doi.org/10.1080/03601234.2013.824236

Gu, H., Zhang, J., Bao, J., 2015. High tolerance and physiological mechanism of Zymomonas mobilis to phenolic inhibitors in ethanol fermentation of corncob residue. Biotechnol Bioeng 112, 1770–1782. https://doi.org/10.1002/bit.25603

Hill, P., Benjamin, K., Bhattacharjee, B., Garcia, F., Leng, J., Liu, C.L., Murarka, A., Pitera, D., Rodriguez Porcel, E.M., da Silva, I., Kraft, C., 2020. Clean manufacturing powered by biology: how Amyris has deployed technology and aims to do it better. J Ind Microbiol Biotechnol 47, 965–975. https://doi.org/10.1007/S10295-020-02314-3

Hoff, B., Plassmeier, J., Blankschien, M., Letzel, A.C., Kourtz, L., Schröder, H., Koch, W., Zelder, O., 2021. Unlocking Nature's Biosynthetic Power—Metabolic Engineering for the Fermentative Production of Chemicals. Angewandte Chemie International Edition 60, 2258–2278. https://doi.org/10.1002/ANIE.202004248

Jacobs, A., Auburger, S., Bahrs, E., Brauer-Siebrecht, W., Christen, O., Götze, P., Koch, H.J., Mußhoff, O., Rücknagel, J., Märländer, B., 2017. Replacing silage maize for

biogas production by sugar beet – A system analysis with ecological and economical approaches. Agric Syst 157, 270–278. https://doi.org/10.1016/J.AGSY.2016.10.004

Kimani, B.G., Kerekes, E.B., Szebenyi, C., Krisch, J., Vágvölgyi, C., Papp, T., Takó, M., 2021. In vitro activity of selected phenolic compounds against planktonic and biofilm cells of food-contaminating yeasts. Foods 10, 1652. https://doi.org/10.3390/FOODS10071652/S1

Koss-Mikołajczyk, I., Kusznierewicz, B., Wiczkowski, W., Sawicki, T., Bartoszek, A., 2019. The comparison of betalain composition and chosen biological activities for differently pigmented prickly pear (Opuntia ficus-indica) and beetroot (Beta vulgaris) varieties. 70, 442–452. https://doi.org/10.1080/09637486.2018.1529148

Kotzamanidis, C., Roukas, T., Skaracis, G., 2002. Optimization of lactic acid production from beet molasses by Lactobacillus delbrueckii NCIMB 8130. World J Microbiol Biotechnol 18, 441–448. https://doi.org/10.1023/A:1015523126741

Lechner, J.F., Stoner, G.D., 2019. Red Beetroot and Betalains as Cancer Chemopreventative Agents. Molecules 24. https://doi.org/10.3390/MOLECULES24081602

Amorin, H., Basso, L., Lopes, M., 2009. Sugar cane juice and molasses, beet molasses and sweet sorghum: composition and usage. The Alcohol Textbook 7, 343–354. https://doi.org/10.2/JQUERY.MIN.JS

Marzo, C., Díaz, A.B., Caro, I., Blandino, A., 2019. Status and Perspectives in Bioethanol Production From Sugar Beet. Bioethanol Production from Food Crops: Sustainable Sources, Interventions, and Challenges 61–79. https://doi.org/10.1016/B978-0-12-813766-6.00004-7

Masih, D., Singh, N., Singh, A., 2019. Red beetroot: A source of natural colourant and antioxidants: A review. J Pharmacogn Phytochem 8, 162–166.

Meadows, A.L., Hawkins, K.M., Tsegaye, Y., Antipov, E., Kim, Y., Raetz, L., Dahl, R.H., Tai, A., Mahatdejkul-Meadows, T., Xu, L., Zhao, L., Dasika, M.S., Murarka, A., Lenihan, J., Eng, D., Leng, J.S., Liu, C.-L., Wenger, J.W., Jiang, H., Chao, L., Westfall, P., Lai, J., Ganesan, S., Jackson, P., Mans, R., Platt, D., Reeves, C.D., Saija, P.R., Wichmann, G., Holmes, V.F., Benjamin, K., Hill, P.W., Gardner, T.S., Tsong,

A.E., 2016. Rewriting yeast central carbon metabolism for industrial isoprenoid production. Nature 537, 694–697. https://doi.org/10.1038/nature19769

Mendes, A., Azevedo-Silva, J., Fernandes, J.C., 2022. From Sharks to Yeasts: Squalene in the Development of Vaccine Adjuvants. Pharmaceuticals 2022, Vol. 15, Page 265 15, 265. https://doi.org/10.3390/PH15030265

Merico, A., Sulo, P., Piškur, J., Compagno, C., 2007. Fermentative lifestyle in yeasts belonging to the Saccharomyces complex. FEBS J 274, 976–989. https://doi.org/10.1111/J.1742-4658.2007.05645.X

Merkl, R., Hrádková, I., Filip, V., Šmidrkal, J., 2010. Antimicrobial and antioxidant properties of phenolic acids alkyl esters. Czech Journal of Food Sciences 28 (2010), 275–279. https://doi.org/10.17221/132/2010-CJFS

Mesquita, T.J.B., Sargo, C.R., Fuzer, J.R., Paredes, S.A.H., Giordano, R.D.C., Horta, A.C.L., Zangirolami, T.C., 2019. Metabolic fluxes-oriented control of bioreactors: A novel approach to tune micro-Aeration and substrate feeding in fermentations. Microb Cell Fact 18, 1–17. https://doi.org/10.1186/S12934-019-1198-6

M'hiri, N., Ioannou, I., Ghoul, M., Boudhrioua, N.M., 2014. Extraction Methods of Citrus Peel Phenolic Compounds. https://doi.org/10.1080/87559129.2014.924139 30, 265–290. https://doi.org/10.1080/87559129.2014.924139

Muir, B.M., Anderson, A.R., 2022. Development and Diversification of Sugar Beet in Europe. Sugar Tech 24, 992–1009. https://doi.org/10.1007/S12355-021-01036-9

Munteanu, I.G., Apetrei, C., 2021. Analytical Methods Used in Determining Antioxidant Activity: A Review. Int J Mol Sci 22. https://doi.org/10.3390/IJMS22073380

Oliveira, A.L.S., Seara, M., Carvalho, M.J., Mota De Carvalho, N., Costa, E.M., Silva, S., Duarte, M., Pintado, M., Oliveira, C., Madureira, A.R., 2023. Production of Sustainable Postbiotics from Sugarcane Straw for Potential Food Applications. Applied Sciences 2023, Vol. 13, Page 3391 13, 3391. https://doi.org/10.3390/APP13063391

Oliveira, A.S., Pereira, J.O., Ferreira, C., Faustino, M., Durão, J., Pintado, M.E., Carvalho, A.P., 2022. Peptide-rich extracts from spent yeast waste streams as a source of

bioactive compounds for the nutraceutical market. Innovative Food Science & Emerging Technologies 81, 103148. https://doi.org/10.1016/J.IFSET.2022.103148

Płatosz, N., Sawicki, T., Wiczkowski, W., 2020. Profile of phenolic acids and flavonoids of red beet and its fermentation products. Does long-term consumption of fermented beetroot juice affect phenolics profile in human blood plasma and urine? Pol J Food Nutr Sci 70, 55–65. https://doi.org/10.31883/PJFNS/116613

Rafiqul, I.S.M., Sakinah, A.M.M., Zularisam, A.W., 2015. Inhibition by toxic compounds in the hemicellulosic hydrolysates on the activity of xylose reductase from Candida tropicalis. Biotechnol Lett 37, 191–196. https://doi.org/10.1007/S10529-014-1672-5/TABLES/2

Ritchie, H., Rosado, P., Roser, M., 2023. Agricultural Production. Our World in Data.

Shi, T., Li, Y., Zhu, L., Tong, Y., Yang, J., Fang, Y., Wang, M., Zhang, J., Jiang, Y., Yang, S., 2021. Engineering the oleaginous yeast Yarrowia lipolytica for β-farnesene overproduction. Biotechnol J 16, 2100097. https://doi.org/10.1002/BIOT.202100097

Shirai, A., Watanabe, T., Matsuki, H., 2017. Inactivation of foodborne pathogenic and spoilage micro-organisms using ultraviolet-A light in combination with ferulic acid. Lett Appl Microbiol 64, 96–102. https://doi.org/10.1111/LAM.12701

Slavov, A., Karagyozov, V., Denev, P., Kratchanova, M., Kratchanov, C., 2013. Antioxidant activity of red beet juices obtained after microwave and thermal pretreatments. 31, 139–147. https://doi.org/10.17221/61/2012-CJFS

Sluiter, A.D., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., Crocker, D.P., 2004. Determination of Structural Carbohydrates and Lignin in Biomass.

Tan, L., Sun, Z.Y., Okamoto, S., Takaki, M., Tang, Y.Q., Morimura, S., Kida, K., 2015. Production of ethanol from raw juice and thick juice of sugar beet by continuous ethanol fermentation with flocculating yeast strain KF-7. Biomass Bioenergy 81, 265–272. https://doi.org/10.1016/J.BIOMBIOE.2015.07.019

Tropea, A., 2022. Biofuels Production and Processing Technology. Fermentation 2022, Vol. 8, Page 319 8, 319. https://doi.org/10.3390/FERMENTATION8070319

Valladares-Diestra, K.K., Porto de Souza Vandenberghe, L., Nishida, V.S., Zevallos Torres, L.A., Zandoná Filho, A., Soccol, C.R., 2022. Integrated sugarcane biorefinery for first- and second-generation bioethanol production using imidazole pretreatment. J Clean Prod 381, 135179. https://doi.org/10.1016/J.JCLEPRO.2022.135179

Valli, V., Gómez-Caravaca, A.M., Di Nunzio, M., Danesi, F., Caboni, M.F., Bordoni, A., 2012. Sugar cane and sugar beet molasses, antioxidant-rich alternatives to refined sugar. J Agric Food Chem 60, 12508–12515. https://doi.org/10.1021/JF304416D

Vučurović, V.M., Puškaš, V.S., Miljić, U.D., 2019. Bioethanol production from sugar beet molasses and thick juice by free and immobilised Saccharomyces cerevisiae. Journal of the Institute of Brewing 125, 134–142. https://doi.org/10.1002/JIB.536

Yao, P., You, S., Qi, W., Su, R., He, Z., 2020. Investigation of fermentation conditions of biodiesel by-products for high production of β-farnesene by an engineered Escherichia coli. Environmental Science and Pollution Research 27, 22758–22769. https://doi.org/10.1007/S11356-020-08893-Z

Chapter 5

Final Remarks

Conclusions

The work in the present thesis showcased the influence of phenolic compounds during the β-farnesene fermentation process with *S. cerevisiae*. Firstly, the sugarcane syrup used in the Amyris fermentation process was characterized for its phenolic content (**Chapter 2**). Hydroxybenzoic acids, hydroxycinnamic acids and flavonoids were the classes of phenolic compounds present in the syrup. Additionally, the TPC of sugarcane syrup was measured to be around 50 mg/L and hydroxycinnamic acids represented half of the TPC. This insight into the phenolic composition of the syrup provided a foundational understanding for subsequent stages of the study and enabled the exploration of the potential impacts of these compounds on the fermentation process and product outcomes. In addition, the investigation into the evolution of phenolic compounds during the 13-days of β-farnesene fed-batch fermentation period unveiled dynamic patterns. Most compounds exhibited an ascending trend in concentration until stabilizing post day 3. Interestingly, certain phenolic compounds exhibited a decline after day 2, implying their potential metabolization by the yeast. This observation highlights the complex interplay between yeast metabolism and phenolic compounds' dynamics during fermentation. The phenolic evolution was also modulated according to two mathematical calculations and the Weibull model demonstrated the superior fit to the experimental data, which underscores this model as an effective predictive tool for the evolution of phenolic compounds in fed-batch fermentations.

After characterization of the phenolic content during the fermentation process, the phenolic compounds from sugarcane syrup were removed with activated charcoal (**Chapter 3**). The optimization of the syrup purification demonstrated that the concentration of charcoal had a positive effect on the phenolic purification, but a negative effect on the syrup recovery yield. The optimized condition was identified as employing the charcoal with the highest surface area at almost the highest concentration, which resulted in 97 % reduction in syrup TPC. To evaluate the effect of the phenolic compound removal, batch fermentations were performed with purified and non-purified syrup. The purified syrup presented

decreased antioxidant activity, leading to higher oxidative stress in the yeast over the fermentation. The β-farnesene productivity varied with the scale of fermentation, with improvements found in shake-flask, but not in bioreactor. Consequently, for the conditions tested, the phenolic compounds impacted on yeast production of β-farnesene at small scale, while their effect appear negligible at large scale.

Then, a feedstock with lower phenolic content was used to produce β-farnesene using the established fermentation process by Amyris. Sugarbeet syrup was utilized both in the fed-batch fermentation process and for the bioreactor steps involved in inoculum preparation (**Chapter 4**). Beet syrup presented lower concentration of phenolic compounds than cane syrup, but higher concentrations of other possible inhibitors, such as glucose and HMF. The slow growth of the inoculum in the second bioreactor step was optimized using a fed-batch process with 40 g/L of initial beet syrup TRS and with pulses of 10 g/L TRS. The production fermentation using beet syrup was successfully conducted, marking the first instance of accomplishing β-farnesene production with this feedstock. A decrease in the content of certain phenolic compounds during the fermentation suggested their metabolization by the yeast. The final farnesene concentration, cumulative farnesene yield and productivity of the fermentations using beet syrup were 127 g/L, 19.44 % and 1.70 g/L/h, respectively. These results collectively highlight the efficacy of employing beet syrup as a feedstock and illustrate the potential for β-farnesene production using this alternative carbon source. This achievement not only expands the spectrum of viable feedstocks but also holds implications for advancing sustainable and efficient β-farnesene production.

Overall, the work from this thesis showed that the phenolic content increased during the β-farnesene fed-batch fermentation process until reaching a stable range. It became possible to predict the phenolic content of the fermentation broth using mathematical calculations, such as the Weibull model. The data corroborate that the yeast *S. cerevisiae* is likely responsible for the metabolization of multiple phenolic compounds during the fermentation, decreasing their concentration. The removal of phenolic compounds from the sugarcane syrup with activated charcoal was successful, leading to a maximum of 97 % reduction in the phenolic content. Notwithstanding this achievement, the utilization of the purified syrup in the fermentation process did not enhance bioreactor productivity; instead, it resulted in only a small improvement in yeast growth. On the other hand, the use of beet syrup, containing lower phenolic content than sugarcane syrup, was performed for the first

time in the production of β-farnesene. The utilization of beet syrup as an alternative feedstock resulted in the successful attainment of high β-farnesene concentration and cumulative yield, thereby confirming the feasibility of employing beet syrup in the β-farnesene production. Lastly, this work has demonstrated that the phenolic compounds have some effects on the bioreactor fermentation process, by supplying antioxidant activity to help combat oxidative stress. Although the presence of phenolic compounds in sugarcane syrup imparts antioxidant activity, it does not appear to significantly impact fermentation parameters, nor does it hinder the fermentation process.

Future Perspectives

This thesis has demonstrated that the phenolic compounds in sugarcane syrup provide antioxidant activity to the fermentation process, and that *S. cerevisiae* has the capability of converting some phenolic compounds. Nevertheless, to enhance the understanding of the effects of phenolic compounds, it would be important to supply purified syrup in fed-batch fermentations, the process used to produce β-farnesene at industrial scale. However, in the context of fed-batch processes, a significantly larger quantity of syrup is required, consequently requiring the increase of both the scale and the complexity of the syrup purification process.

During the removal of phenolic compounds from the sugarcane syrup with activated charcoal, the mineral content of the syrup was slightly altered. Activated charcoal was selected in this thesis due to its ease of use, availability, and cost. However, other methodologies for the specific removal of phenolic compounds from the sugarcane syrup, such as ion-exchange resin beads, may be employed to understand if the purification process with activated charcoal reduced the content of essential nutrients for the yeast fermentation. In addition, this method may allow a superior recovery of syrup mass.

In case phenolic compounds show to affect the fed-batch fermentation process, genetic engineering of *S. cerevisiae* may also be performed to improve yeast tolerance to phenolic compounds during fermentation. Multiple genetic modifications have been described in the literature, such as the overexpression of endogenous genes coding for phenylacrylic acid decarboxylase or for ALD5, ATF1 and ATF2 (alcohol acetyltransferases). These enzymes have been shown to convert cinnamic, *p*-coumaric and ferulic acids, which can help improve yeast growth, thus showing potential for improving the fermentation process.

Furthermore, while the implementation of beet syrup as an alternative feedstock proved successful, opportunities for further improvements still exist. For instance, the enhancement of the fermentation process may be conducted by using a beet syrup resulting from a manufacturing process that does not produce degradation of the sucrose molecule into its monomers nor the generation of HMF. Decreased levels of glucose and HMF may reduce the inhibition caused by these molecules, either by the Crabtree effect or sugar intake and protein synthesis, respectively. In turn, the absence of inhibition by these molecules may

help to enhance β-farnesene productivity and improve the fermentation's economic profitability.