Universidade do Minho
Escola de Engenharia

Carlos Ezequiel Antunes Costa

Resveratrol *de novo* biosynthesis from lignocellulosic biomass: metabolic engineering of thermotolerant robust yeast strains for an integrated and intensified bioprocess

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Doctoral Thesis
Doctoral Program in Chemical and Biological Engineering

Work developed under the supervision of
Prof. Doctor Lucília Maria Alves Ribeiro Domingues
Prof. Doctor José António Couto Teixeira
Doctor Aloia Romaní Pérez

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Uma página é muito pouco para expressar toda a gratidão que cabe em mim, e tão pouco me chegaria a dimensão desta tese, na sua totalidade, para a manifestar. Como até aqui, darei o meu melhor.

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STATEMENT OF INTEGRITY

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

Resumo

Biossíntese de resveratrol a partir de biomassa lignocelulósica: engenharia metabólica de leveduras robustas e termotolerantes para um processo integrado e intensificado

Microrganismos robustos são essenciais para desenvolver processos sustentáveis e industrialmente atrativos. A levedura *Saccharomyces cerevisiae* é amplamente utilizada como fábrica celular para produzir biocombustíveis e outros bioprodutos de alto valor, sendo as estirpes industriais conhecidas pela sua elevada capacidade fermentativa e aptidão para lidar com condições adversas (p.e. altas temperaturas, baixo pH). O resveratrol é um composto polifenólico antioxidante, geralmente extraído de plantas ou sintetizado quimicamente, processos considerados complexos e não sustentáveis. A sua biossíntese pode ser uma alternativa valiosa para compensar estes inconvenientes, embora seja geralmente obtida à custa de substratos dispendiosos como o ácido *p*-cumárico. A produção *de novo* de resveratrol a partir de fontes de carbono pode, portanto, ser crucial para ultrapassar estes obstáculos.

Nesta tese, várias estirpes industriais de *S. cerevisiae* foram geneticamente modificadas através de CRISPR/Cas9 com uma via biossintética de resveratrol (VBR). Após avaliação das estirpes recombinantes em fermentação até 39 ºC, a estirpe mais termotolerante (Ethanol Red) foi aplicada na produção de resveratrol por Sacarificação e Fermentação Simultâneas de madeira de eucalipto pré-tratada hidrotermicamente, sendo obtida uma concentração de 152 mg/L. Depois, a utilização de lactose foi viabilizada pela expressão heteróloga de uma permease de lactose e de uma β-galactosidase. A estirpe resultante metabolizou eficientemente altas concentrações de lactose e, após a otimização das condições de oxigenação, foi atingido um título de 284 mg/L de resveratrol. Esta estirpe foi igualmente capaz de produzir resveratrol a partir de soro de queijo. A VBR foi também expressa numa estirpe Ethanol Red que metaboliza xilose, com os genes da fase não-oxidativa da via das pentoses fosfato sobre-expressos, melhorando o fornecimento de precursores da VBR. Após otimização da atividade do citocromo P450 e da suplementação do meio, esta estirpe produziu 224 mg/L de resveratrol a partir da xilose. A co-fermentação simultânea de glucose e xilose levou a um título de resveratrol de 388 mg/L, 1,35 vezes maior do que em glucose para a mesma molaridade total de carbono. Esta estirpe foi utilizada para a produção de resveratrol na valorização de resíduos da indústria vinícola como o resíduo de poda da vinha (glucose/xilose), o mosto de uva (glucose/frutose) e as borras de vinho (etanol). Os resultados aqui apresentados contribuem para alargar a aplicação do conceito de biorrefinaria, baseada em resíduos agroindustriais, na produção de compostos-alvo de maior valor, destacando o papel da *S. cerevisiae* no desenvolvimento de bioprocessos mais ecológicos, seguindo o conceito de bioeconomia circular.

Palavras-chave: Biomassa lignocelulósica, Biorrefinaria, CRISPR/Cas9, Levedura, Resveratrol.

Abstract

Resveratrol *de novo* biosynthesis from lignocellulosic biomass: metabolic engineering of thermotolerant robust yeast strains for an integrated and intensified process

Robust microorganisms are essential to develop sustainable and industrially attractive processes. The yeast *Saccharomyces cerevisiae* is widely used as a cell factory to produce biofuels and other high-value natural products, industrial strains being known for their high fermentative capacity and ability to cope with harsh conditions (e.g. high temperatures, low pH). Resveratrol is an antioxidant polyphenolic compound, generally extracted from plants or chemically synthesised, both processes considered complex and non-sustainable. Its biosynthesis can be a valuable alternative to offset these drawbacks, though it is generally achieved at the cost of expensive substrates like *p*-coumaric acid. *De novo* resveratrol production from carbon sources can, therefore, be crucial to overcome these hindrances.

In this thesis, a set of robust industrial yeast strains, known for their excellent fermentative performance, was engineered via the CRISPR/Cas9 system with a resveratrol biosynthetic pathway (RBP). After screening the recombinant strains in fermentation up to 39 ºC, the most thermotolerant yeast strain, an Ethanol Red-derivative, was applied for resveratrol production by Simultaneous Saccharification and Fermentation of hydrothermally pretreated eucalyptus wood, achieving a titre of 152 mg/L. Subsequently, lactose utilisation was enabled by the heterologous expression of a lactose permease and a β-galactosidase. The resultant strain was able to efficiently metabolise a high concentration of lactose and, after fine-tuning the oxygenation conditions, 284 mg/L of resveratrol was attained. This strain was also capable of producing resveratrol using exclusively cheese whey as a substrate. The RBP was also expressed in a xylose-consuming Ethanol Red strain, with an overexpression of the non-oxidative pentose phosphate pathway genes, improving the precursor supply of the RBP. After enhancing the cytochrome P450 activity and optimising the supplementation of the media, this strain was able to produce 224 mg/L of resveratrol from xylose. Simultaneous co-fermentation of glucose and xylose led to a resveratrol titre of 388 mg/L, 1.35-fold higher than in glucose alone with the same total carbon molarity. This strain was used for the valorisation of agro-industrial wastes from the wine industry, a source of several fermentable sugars, namely glucose and xylose (vine pruning residue), glucose and fructose (grape must) and ethanol (wine lees). Altogether, the results displayed in this thesis contribute to the expansion of possible target chemicals for a biorefinery based on different agro-industrial by-products, highlighting the role of robust industrial *S. cerevisiae* strains for the establishment of greener bioprocesses following the circular bioeconomy concept.

Keywords: Biorefinery, CRISPR/Cas9, Lignocellulosic biomass, Resveratrol, Yeast

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List of abbreviations and acronyms

4CL	4-Coumarate:CoA Ligase
ANOVA	Analysis Of Variance
AR	Aldose Reductase
ATP	Adenosine Triphosphate
BDW	Biomass Dry Weight
BSA	Bovine Serum Albumin
C4H	Cinnamate 4-Hydroxylase
CAGR	Compound Annual Growth Rate
CHI	Chalcone Isomerase
CHS	Chalcone Synthase
CoA	Coenzyme A
COVID-19	Coronavirus Disease 2019
CRISPR	Clustered Regularly Interspaced Palindromic Repeats
CRISPRi	CRISPR Interference
CVD	Cardiovascular Disease
CWP	Cheese Whey Powder
DAHP	3-deoxy-D-arabinoheptulosonate 7-phosphate
DES	Deep Eutectic Solvent
DNA	Deoxyribonucleic Acid
DSB	Double-Strand Break
DW	Dry Weight
E4P	Erythrose 4-phosphate
EGW	*Eucalyptus globulus* wood
FAO	Food and Agriculture Organization of the United Nations
FPU	Filter Paper Units
Fru1,6P	Fructose 1,6-biphosphate
Fru1P	Fructose 1-phosphate
Fru6P	Fructose 6-phosphate
FW	Fresh Weight
GA	Glyceraldehyde

GA3P	Glyceraldehyde 3-phosphate
Glu6P	Glucose 6-phosphate
GM	Grape Must
GMO	Genetically Modified Organism
GRAS	Generally Regarded As Safe
gRNA	Guide RNA
HMF	5-Hydroxymethylfurfural
HPLC	High-Performance Liquid Chromatography
HR	Homologous Recombination
IGF-1	Insulin-like Growth Factor 1
IGFB-3	Insulin-like Growth Factor-Binding protein 3
LB	Lysogeny Broth
LCM	Lignocellulosic Material(s)
LDL	Low-density Lipoprotein
LSR	Liquid Solid Ratio
NAD^+	Oxidised Nicotinamide Adenine Dinucleotide
NADH	Reduced Nicotinamide Adenine Dinucleotide
$NADP^+$	Oxidised Nicotinamide Adenine Dinucleotide Phosphate
NADPH	Reduced Nicotinamide Adenine Dinucleotide Phosphate
OD_{600}	Optical Density at 600 nm
OIV	International Organisation of Vine and Wine
PAL	Phenylalanine Ammonia Lyase
PAM	Protospacer Adjacent Motif
PCR	Polymerase Chain Reaction
pdCWP	Partially Deproteinised Cheese Whey Powder
PEG	Polyethylene Glycol
PEP	Phosphoenolpyruvate
PPP	Pentose Phosphate Pathway
RASSF-1α	Ras Association domain Family 1α
RBP	Resveratrol Biosynthetic Pathway
RNA	Ribonucleic Acid
Ro5P	Ribose 5-phosphate

Ru5P	Ribulose 5-phosphate
S_0	Severity
S7P	Sedoheptulose 7-phosphate
SARS-CoV-2	Severe Acute Respiratory Syndrome Coronavirus 2
SD	Standard Deviation
SHF	Separate Hydrolysis and Fermentation
SIRT-1	Sirtuin Type 1
SSF	Simultaneous Saccharification and Fermentation
STS	Stilbene Synthase
TAL	Tyrosine Ammonia Lyase
UHPLC	Ultra-High-Performance Liquid Chromatography
USER	Uracil-Specific Excision Reagent
UV	Ultraviolet
VPR	Vine Pruning Residue
VST	Resveratrol Synthase
WL	Wine Lees
X5P	Xylulose 5-phosphate
XDH	Xylitol Dehydrogenase
XI	Xylose Isomerase
XK	Xylulokinase
XR	Xylose Reductase
YE	Yeast Extract
YED	Yeast Extract, Dextrose
YEDX	Yeast Extract, Dextrose, Xylose
YPD	Yeast extract, Peptone, Dextrose
YPL	Yeast extract, Peptone, Lactose
YPX	Yeast extract, Peptone, Xylose

Scientific outputs

According to the 2nd paragraph of article 8 of the Portuguese Decree-Law no. 388/70, the scientific outputs of this thesis are listed below.

The results presented in this thesis have been partially published elsewhere.

Peer-reviewed journal articles:

Cunha JT, Romaní A, Costa CE, Sá-Correia I, Domingues L. **2019**. Molecular and physiological basis of *Saccharomyces cerevisiae* tolerance to adverse lignocellulose-based process conditions. *Appl. Microbiol. Biotechnol. 103* (1). 159–175. DOI: 10.1007/s00253-018-9478-3.

Cunha JT, Soares PO, Baptista SL, Costa CE, Domingues L. **2020**. Engineered *Saccharomyces cerevisiae* for lignocellulosic valorization: a review and perspectives on bioethanol production. *Bioengineered. 11* (1). 883–903. DOI: 10.1080/21655979.2020.1801178.

Lip KYF, García-Ríos E, Costa CE, Guillamón JM, Domingues L, Teixeira J, van Gulik WM. **2020**. Selection and subsequent physiological characterisation of industrial *Saccharomyces cerevisiae* strains during continuous growth at sub- and- supra optimal temperatures. *Biotechnol. Reports. 26*. e00462. DOI: 10.1016/j.btre.2020.e00462.

Costa CE, Møller-Hansen I, Romaní A, Teixeira JA, Borodina I, Domingues L. **2021**. Resveratrol production from hydrothermally pretreated eucalyptus wood using recombinant industrial *Saccharomyces cerevisiae* strains. *ACS Synth. Biol. 10* (8). 1895–1903. DOI: 10.1021/acssynbio.1c00120.

Baptista SL*****, Costa CE*****, Cunha JT, Soares PO, Domingues L. **2021**. Metabolic engineering of *Saccharomyces cerevisiae* for the production of top value chemicals from biorefinery carbohydrates. *Biotechnol. Adv. 47*. 107697. DOI: 10.1016/j.biotechadv.2021.107697.

Carvalho P*****, Costa CE*****, Baptista SL, Domingues L. **2021**. Yeast cell factories for sustainable whey-to-ethanol valorisation towards a circular economy. *Biofuel Res. J. 8* (4). 1529–1549. DOI: 10.18331/BRJ2021.8.4.4.

Costa CE, Romaní A, Teixeira JA, Domingues L. **2022**. Resveratrol production for the valorisation of lactose-rich wastes by engineered industrial *Saccharomyces cerevisiae. Bioresour. Technol.* (in press)

***** *Authors contributed equally to this work*

Submitted manuscripts:

Costa CE*, Carvalho P*, Domingues L. Strategic combination of different promoters in lactose metabolisation and host chassis selection for high bioethanol titres from dairy wastes.

***** *Authors contributed equally to this work*

Manuscripts in preparation:

Costa CE, Romaní A, Møller-Hansen I, Teixeira JA, Borodina I, Domingues L. Valorisation of wine wastes by *de novo* biosynthesis of resveratrol using a recombinant xylose-consuming industrial *S. cerevisiae.*

Posters in conferences:

Costa CE, Møller-Hansen I, Borodina I, Domingues L. **2019**. Robust industrial strains as platform for *de novo* resveratrol production from carbon sources: establishing grounds for an integrated process. Microbiotec'19 - Congress of Microbiology and Biotechnology. Coimbra, Portugal.

Costa CE, Møller-Hansen I, Romaní A, Teixeira JA, Borodina I, Domingues L. **2021**. Resveratrol production by recombinant robust yeast strains for valorisation of wine waste. Microbiotec'21 - Congress of Microbiology and Biotechnology. Lisbon, Portugal.

Costa CE, Carvalho P, Baptista SL, Domingues L. **2022**. Evaluation of efficient metabolic engineering strategies for accelerated co-utilisation of lactose by *Saccharomyces cerevisiae.* 3rd BioIberoAmerica – Ibero-American Congress on Biotechnology. Braga, Portugal.

Oral communications in conferences:

Costa CE, Møller-Hansen I, Borodina I, Domingues L. **2018**. Metabolic engineering of robust industrial strains for *de novo* resveratrol production from sole carbon sources for application in process-like conditions. 4th Applied Synthetic Biology in Europe. Toulouse, France.

Costa CE, Møller-Hansen I, Romaní A, Teixeira JA, Borodina I, Domingues L. **2022**. Engineered industrial yeast for resveratrol de novo production from wine waste. 3rd BioIberoAmerica – Ibero-American Congress on Biotechnology. Braga, Portugal.

Thesis outline

The research activities resulting in this doctoral thesis were mainly developed at CEB – Centre of Biological Engineering, University of Minho (Braga, Portugal) under the supervision of Professor Lucília Domingues, Professor José Teixeira and Doctor Aloia Romaní. This doctoral thesis is divided into five chapters, three of them describing experimental research published or in preparation for submission to international peer-reviewed journals.

Chapter I encompasses a comprehensive review of the development of a biorefinery based on lignocellulosic biomass and industrial by-products and its application to phenylpropanoids biosynthesis, focusing on resveratrol, its properties, and bioprocesses. Additionally, the state of the art of genetic engineering strategies is approached, detailing the novel genome-editing technology CRISPR/Cas9, and tools for its application in the yeast *Saccharomyces cerevisiae*.

Chapter II focuses on the selection of suitable hosts for resveratrol biosynthesis from glucose and ethanol. Different *S. cerevisiae* strains were engineered with the Resveratrol Biosynthetic Pathway via CRISPR/Cas9 and screened for their aptitude to produce resveratrol at different temperatures (30 and 39 ºC). Resveratrol production from hydrothermally pretreated *Eucalyptus globulus* wood by Simultaneous Saccharification and Fermentation was also accomplished. The metabolic engineering tasks displayed in this chapter were performed by the author of this thesis at The Novo Nordisk Foundation Center for Biosustainability (Kgs. Lyngby, Denmark), under the supervision of Professor Irina Borodina and Doctor Iben Møller-Hansen.

Chapter III explores resveratrol production from lactose, a disaccharide non-naturally metabolised by *S. cerevisiae*. Lactose metabolisation was achieved by heterologous expression of a lactose permease and β-galactosidase in a previously selected yeast strain. Process optimisation by fine-tuning oxygenation conditions was developed for increased titres and using cheese whey as a renewable carbon source for fermentation.

Chapter IV focuses on the use of multiple carbon sources for the valorisation of agro-industrial wastes from the wine industry. Resveratrol *de novo* production from xylose using engineered *S. cerevisiae* was demonstrated, as well as the positive effect of the Pentose Phosphate Pathway and enhancement of the cytochrome P450 activity in resveratrol production. After optimisation of media supplementation for increased titres, benefits from simultaneous utilisation of glucose and xylose were shown. The hemicellulosic fraction of vine pruning residue, wine lees and grape must, residues from the wine industry, were efficiently used as substrates for resveratrol production.

Chapter V offers the major conclusions of this thesis, emphasising its contribution to the development of resveratrol processes within a biorefinery scheme, alongside future perspectives for further developments on the topic.

Chapter I.
General Introduction

This chapter is partially based on the following review articles:

Baptista SL*****, Costa CE*****, Cunha JT, Soares PO, Domingues L. **2021**. Metabolic engineering of *Saccharomyces cerevisiae* for the production of top value chemicals from biorefinery carbohydrates. *Biotechnol. Adv. 47*. 107697. DOI: 10.1016/j.biotechadv.2021.107697.

Carvalho P*****, Costa CE*****, Baptista SL, Domingues L. **2021**. Yeast cell factories for sustainable whey-to-ethanol valorisation towards a circular economy. *Biofuel Res. J. 8* (4). 1529–1549. DOI: 10.18331/BRJ2021.8.4.4.

Cunha JT, Romaní A, Costa CE, Sá-Correia I, Domingues L. **2019**. Molecular and physiological basis of *Saccharomyces cerevisiae* tolerance to adverse lignocellulose-based process conditions. *Appl. Microbiol. Biotechnol. 103* (1). 159–175. DOI: 10.1007/s00253-018-9478-3.

Cunha JT, Soares PO, Baptista SL, Costa CE, Domingues L. **2020**. Engineered *Saccharomyces cerevisiae* for lignocellulosic valorization: a review and perspectives on bioethanol production. *Bioengineered. 11* (1). 883–903. DOI: 10.1080/21655979.2020.1801178.

***** *Authors contributed equally to this work*

1.1. The biorefinery concept

In a broad sense, biorefining is described as the sustainable processing of biomass into a range of marketable biobased products and bioenergy.[1] The biorefinery concept comprehends the use of a spectrum of technologies to convert renewable resources, such as lignocellulosic biomass, cheese whey or other agro-industrial wastes into the respective building blocks that can be used for the production of biofuels, chemicals or other value-added compounds.[2]

Contrary to the petroleum-based refinery, where natural resources are largely exploited with tremendous waste generation, biorefinery embodies a major shift by integrating systems that enable full resource usage.[2] The establishment of a biorefinery fulfils two main purposes: an energy goal, which is driven by the need for renewable energy sources; and an economic goal, focusing on the development of a biobased industry capable of generating profit.[3]

Although the energy goal is addressed by the efforts made in the biofuel industry, fuel is considered a low-value product. Despite the high-volume production of biofuels, it has limited returns on the funding needed to establish a biorefinery, becoming a barrier to achieving the economic goal.[4] Therefore, a biorefinery able to complement biofuel production with high-value biobased products can effectively aid in the reduction of non-renewable fuel consumption and simultaneously deliver the economic incentive to expand the biorefining industry.[5]

Lasure and Zhang[6] proposed that the biorefinery future would be based on the conversion of lignocellulosic biomass into an array of useful products, where raw materials are separated into different components that can be converted into a target compound. Lignocellulosic biomass is the most available renewable resource on the planet, and it is considered an alternative to fossil carbon sources. Lignocellulosic biomass is composed of cellulose, hemicellulose and lignin. Pretreatment is an essential step to break down its recalcitrant structure, enhancing the enzymatic access to cellulose, being considered the first stage of a biorefinery.[7,8] After the pretreatment, different sugars are obtained, which can serve as building blocks for the bioproduction of a wide range of metabolites of interest using a given microorganism. However, this leads to the formation of inhibitory compounds like weak acids, furans and phenolic compounds.[9,10] In this sense, some requirements are mandatory for an integrated sustainable bioconversion process: (1) identification of tolerance determinants;[11–13] (2) (over)expression of genes involved in tolerance response;[14–16] (3) utilisation of a robust microorganism able to cope with these stress factors.[17–19] Different biorefinery concepts comprising bioethanol production have been proposed[20–24] and significant advances have been made for the overall valorisation of lignocellulosic biomass.

From an industrial perspective, microbial production of biofuels and chemicals has been receiving increased interest, as it allows the use of renewable feedstocks and subsequent production of building blocks at a lower cost than by traditional routes.[25] Among the compounds targeted by the chemical industry, some are not naturally produced by microorganisms or are produced with low yields and titres, with an accumulation of by-products throughout the process. Advances in biotechnological production of chemicals and biofuels are motivated by innovative strategies of genetic engineering, encompassing DNA technology breakthroughs, which enable the creation of superior cell factories.[26] An industrial cell factory must comprise commercial requirements for yield, productivity and titre.[27] A cell factory can either be used for *de novo* synthesis, involving complex metabolic pathways to produce a compound from a simple molecule (e.g. glucose), or for biotransformation, in which a specific reaction produces a compound structurally similar to the substrate molecule.

1.1.1. Lignocellulosic materials

Lignocellulosic biomass is the most available renewable resource on Earth, with a production of around 181.5 thousand million tonnes per year,[28] and may be used to produce liquid biofuels, such as bioethanol,[8,19,22,29] as well as several other high-value chemicals,[30–32] with the added benefit of not competing for land for food production. The sources of lignocellulosic biomass include energy crops, forest biomass, and wastes and agricultural residues.[33]

The intricate and resistant three-dimensional structure of lignocellulosic materials (LCM) is constituted of cellulose, hemicellulose, and lignin. Depending on the biomass source, the composition of each fraction and the structural arrangement between fractions can change, as will the monomer sugar accessibility.[34] Lignin is a complex, highly branching polyphenolic polymer that is mostly found in the cell walls of hardwoods, softwoods and agricultural residues, conferring these plants their rigidity. Up to 75% of lignocellulosic biomass consists of cellulosic and hemicellulosic fractions, which are the primary carbon sources of a biorefinery platform for biofuels and products with added value.[35] Cellulose, a homopolymer of D-glucose, can account for up to 50% of the entire lignocellulosic biomass.[36] Due to the strong hydrogen connections between glucose molecules, its crystalline matrix structure makes it resistant to depolymerisation and insoluble in water.[37] By its turn, hemicellulose is an amorphous heteropolymer composed of short, linear, and branching chains of hexoses (glucose, galactose, and mannose) and pentoses (xylose and arabinose). The backbone of hemicellulose is formed primarily of xylan (-1,4-linked xylose residues), which may account for up to 50% of the composition in certain grass and cereal tissues.[35]

The steps for the utilisation of LCM for biotechnological processes comprise an initial pretreatment to break the recalcitrant structure of lignocellulose, followed by hydrolysis of cellulose and hemicellulose to obtain fermentable sugars and further microbial fermentation of these sugars (Figure **1.1**).[38] Lignocellulosic biomass can be submitted to physic, chemical and/or biological pretreatments to disrupt lignin-cellulose-hemicellulose complexes, resulting in the elimination of lignin, a decrease in cellulose crystallinity, and an increase in the surface area and porosity of the biomass for the hydrolytic enzymes to access, which significantly increases the complexity and time required to extract fermentable sugars. Among the vast variety of pretreatments, hydrothermal treatment (also known as autohydrolysis or liquid hot water) is considered an environmentally friendly pretreatment since it uses water as a reaction medium. Hydrothermal treatment has been widely used for lignocellulosic biomass fractionation due to high selectivity for hemicellulose solubilisation into oligosaccharides and enhancement of enzymatic saccharification of cellulose.[39] Nevertheless, degradation compounds are also generated during hydrothermal treatment, being inhibitors of bioconversion processes.[10,40] The concentration of inhibitory compounds is dependent on the pretreatment severity and has a negative impact on the following saccharification and fermentation steps.[41] These inhibitory compounds are weak acids, furans, namely furfural and hydroxymethylfurfural (HMF), and phenolic compounds.

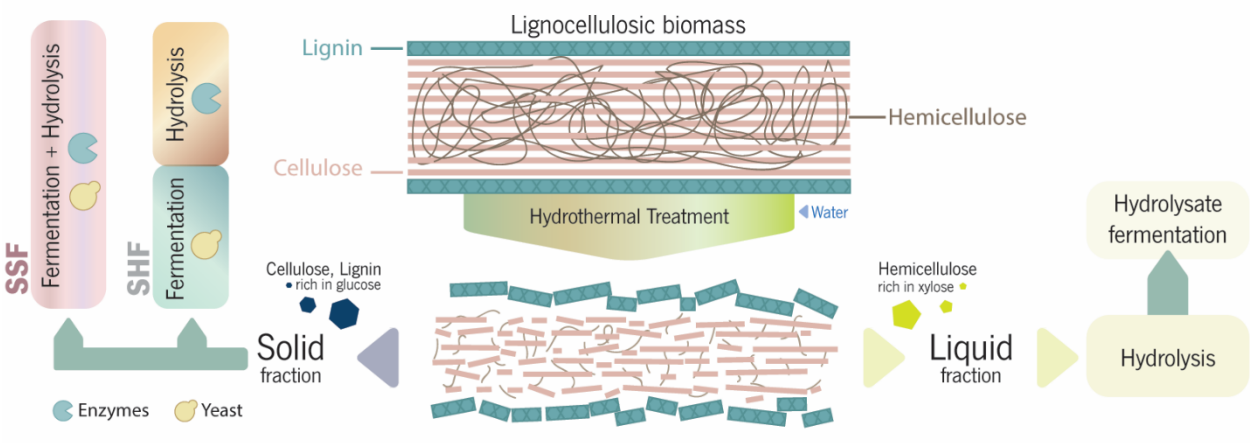

Figure 1.1. Overview of the main steps for the production of biofuels and/or value-added compounds from lignocellulosic materials. The pretreatment breaks the recalcitrant structure of lignocellulose, followed by hydrolysis of cellulose and hemicellulose to fermentable sugars and microbial fermentation. SSF, simultaneous saccharification and fermentation; SHF, separate hydrolysis and fermentation.

Acetic acid is the most abundant weak acid in lignocellulosic hydrolysates and is present due to the deacetylation of acetyl groups linked to the main chain of hemicelluloses. Other weak acids, such as formic and levulinic acids, can also be present in hydrolysates resulting from furan compound degradation.[37] Furfural and HMF are produced by dehydration of pentoses and hexoses, respectively. On

the other hand, phenolic compounds, such as syringic acid, vanillin, ferulic acid, and coumaric acid, are produced by the depolymerisation of lignin. The amount of inhibitory compounds in the lignocellulosic hydrolysates is also dependent on the lignocellulosic source (e.g., agricultural residues, hardwoods, or softwoods), the selected pretreatment (hydrothermal treatment, diluted acid treatment, alkali treatment), and operational conditions (solid loading of lignocellulosic biomass, temperature, time, percentage of catalyst).[22,41] These inhibitory compounds can affect the fermentative performance of microorganisms such as *Saccharomyces cerevisiae* either individually or in combination with each other, and impact the specific growth rate, length of the lag phase, cell viability and vitality and diminish the product yield.[42] Additionally, the concentration of hemicellulose-derived sugars (xylose and xylo-oligosaccharides) can also vary depending on the raw material and the conditions of the LCM pretreatment.

To obtain fermentable sugars, the cellulosic (solid) and hemicellulosic (liquid) fractions arising from pretreatment must be subjected to a hydrolysis process, which is often carried out with the addition of acid catalysts or enzymes. The principal disadvantage of acid hydrolysis is the need to use acids prior to fermentation.[34,35] Regarding the alternative enzymatic hydrolysis, its defining features are the enzyme-substrate specificity, low operation temperatures, and the production of a few inhibitors, making it the most promising and efficient method. On the other hand, enzymatic hydrolysis is hindered by high enzyme costs and yields that fall short of theoretical values[34,43] Taking into mind the constraints associated with pretreatment and hydrolysis procedures, cost-effective utilisation of lignocellulosic biomass for biofuels and value-added products requires a strong microbe to carry out the fermentation process.

Moreover, for an efficient conversion of cellulose to glucose, higher fermentation temperatures are desirable in order to facilitate simultaneous saccharification and fermentation (SSF) processes, as enzymatic saccharification is optimal near 50 ºC, which represents an additional stress factor for the yeast.[44] SSF processes can overcome the drawbacks associated with the alternative separate hydrolysis and fermentation (SHF), by eliminating or reduction of feedback inhibition provoked by the accumulation of sugar monomers on hydrolytic enzymes.[34] In addition, the use of all sugars contained in the hemicellulosic fraction is necessary for the economical utilisation of LCM. Xylose is the most common sugar in the hemicellulosic fraction; yet, microbes such as *S. cerevisiae*, frequently favoured for lignocellulosic ethanol fermentation, are incapable of naturally metabolising this pentose sugar. The aforementioned conditions, necessary for cost-effective lignocellulose-based bioprocesses, combined with the presence of inhibitory chemicals aggravate the unfavourable effects on lignocellulosic fermentation performance, making it crucial to select a robust microorganism to cope with these circumstances.

1.1.2. Dairy industry waste streams

The dairy industry sector is the main source of liquid waste in Europe, essentially due to the disposal of cheese whey.[45] Cheese whey is the primary by-product of cheese production in the dairy industry, consisting of the watery component of milk left after coagulation, being devoid of fat and casein. Riboflavin (vitamin B12) is responsible for the yellow hue of cheese whey, which has a high nutritional value.[46] For every kilogram of cheese that is manufactured, 9 litres of cheese whey are created. The global production of whey from the cheese industry reached 190 million tonnes in 2016, and it is anticipated that its production would expand at the same rate as milk production, 2% per year.[46] Most of the excess cheese whey is currently used for animal feed or land application, posing an environmental hazard. Cheese whey affects the composition of the soil and causes an excess of oxygen consumption, impermeabilization, eutrophication, and toxicity in the area where it is disposed. This impairs plant development and reduces agricultural output.[47,48] Therefore, recycling and reusing waste streams is driven by the growing awareness of environmental issues and the growing need for sustainability.[49]

Several studies have examined the valorisation of cheese whey through the use of technology to recover valuable compounds, such as lactose, proteins, and derived compounds with various applications, or the use of biological processes to produce value-added products, such as ethanol, organic acids, single-cell proteins, enzymes, and proteins.[50,51] Efforts to maximise the value of cheese whey have been propelled by the need to create biorefineries outside of the food sector. Cheese whey processing includes a protein fractionating step that originates the permeate, a lactose-reach stream. This fraction contains 70% of the total solids of whey and still represents an environmental problem. Whey permeate has several potential applications like its use directly in human nutrition, to produce functional beverages or as a substrate for biotechnological processes.[50,52]

Cheese whey and whey permeate comprise a substantial quantity of sugar (almost 50 g/L lactose) and are among the most significant (and unavoidable) industrial waste streams. Cheese whey is mainly composed of water (95% of total whey volume), lactose (70-72% of total solids), whey proteins (8-10% of total solids) and minerals (12-15% of total solids).[46] Moreover, cheese whey has been used as a supplement in yeast fermentation due to its acknowledged nutritional benefits.[53] Cheese whey has also found its way into multi-waste valorisation approaches, in particular, with LCM as substrate, with recent studies exploring its integration in yeast-based processes to increase the overall concentration of the carbon source, increasing ethanol titres with a consequent decrease in distillation costs, in addition to enhancing yeast cell metabolism and reducing water usage.[32,54] Lactose-rich cheese whey and its derivatives have, therefore, high potential for the establishment of biotechnological processes.

1.1.3. Winery by-products

The global agriculture and agro-industrial sectors have conferred significant importance to the wine industry. Grapes are one of the most significant fruit crops worldwide, with an average estimated production of around 75 million tonnes of grapes yearly.[55] As of 2020, an estimated area of 7.3 million ha was planted with grapevines (including young vines not yet in production), and around 262 million hectolitres (mhL) of wine were produced worldwide, with Europe assuming itself as the leading producer of grapes.[56] More specifically, the Iberian Peninsula is an exceptional territory to produce a wide variety of wines, with both Portugal and Spain being amongst the main wine producers on the planet, with 6.4 mhL and 40.7 mhL produced in 2020, respectively, according to the latest report from the International Organisation of Vine and Wine (OIV).[56] The extensive wine manufacture generates large quantities of residues, consisting mainly of grape marc, skin, stalk, and pomace. In addition to these waste products, wineries produce a large amount of wastewater, wine lees and vine pruning residues, and different filtering residues, all of which contribute to environmental deterioration.[57] It is estimated that approx. 10 kg of grapes are necessary to produce 1 L of wine,[55] and that per each kilogram of grape pressed and directed to wine fermentation, more than 20% is disposed of as a waste.[58]

The necessary pruning of the grapevines inevitably leads to high quantities of residues. It is estimated that around 5 tonnes of vine pruning residues (commonly referred to as vine shoots) are generated per hectare of cultivation every year.[59] In Portugal alone, more than 200 thousand hectares are cultivated with grapevines annually, generating high quantities of vine-pruning residue.[60] These are often discarded in agriculture fields due to their low potential to be reutilised for other purposes, such as energy production. Due to its chemical composition, availability, and low cost, this hardwood can be processed as an LCM, as discussed above in the section 1.2.1., and valorised as a feedstock for a wide range of processes.[60,61]

Another valuable feedstock for the establishment of bioprocesses is grape must. It corresponds to the grape juice prior to fermentation, therefore, rich in sugars, namely glucose and fructose, with a total sugar concentration ranging between 180 and 220 g/L, fluctuating depending on cultivar varieties and environmental conditions, with frequent glucose/fructose ratios between 0.91 and 0.99.[62] It can also contain several other substances of interest like magnesium, which is often added to fermentation media.[62] The European Union has implemented legal regulations to minimise wine surplus [Council Regulation (EC) No 1493/1999; Council Regulation (EC) No 479/2008], due to the commercial imbalance that it causes. This excess is managed mostly through regulated distillation and storage for table wine and grape juice,[63,64] in addition to low-quality grape musts, produced in large volumes, which

are not used for wine production. Therefore, its repurpose and use as a substrate for biotechnological processes could aid in the mitigation of the resultant commercial and economic issues.[65]

Wine lees are created during the wine fermentation and ageing processes, collected after the winemaking process has been clarified, and defined as the residue accumulated at the bottom of the wine fermentation vessels or other wine-containing recipients.[66]. Wine lees are constituted by a solid phase, mostly consisting of residual fermentative microorganisms (yeast and bacteria), insoluble polysaccharides and phenolic compounds, among other components, and a liquid phase rich in organic acids and ethanol.[59] They represent between 3.5 and 8.5% of the total amount of by-products generated from grapes during wine manufacturing processes. Generally, wine lees are discarded with wastewater, and while its use as animal feed was previously suggested, its low nutritive value for this purpose makes it unfeasible.[67] However, its application as a substitute nitrogen source shows potential and could bypass the dependency on standard commercial supplementation like yeast extract; even though their pretreatment and application methods should be improved. Additionally, its high content in ethanol brings additional value as a substrate for bioprocesses requiring ethanol as a carbon source, being one of the few agro-industrial wastes containing ethanol in its composition. Therefore, from an economic standpoint, it is reasonable to consider the industrial use of wine lees.

1.2. *S. cerevisiae* as a robust chassis for high-value compound biosynthesis

The yeast *S. cerevisiae*, the popular preferred choice for ethanol fermentation, was the first eukaryotic organism to be sequenced, since then becoming a well-known model organism for the research of eukaryotic functional genomics. Because of its GRAS (Generally Regarded as Safe) status, this yeast may be used to make cultured components for food, cosmetics, and drinks, making waste disposal and product and process approval easier. It is used in quotidian fermentation processes like bread, beer or wine fermentation for centuries, and was the first genetically modified organism (GMO) approved for the manufacture of food additives[68] and the first GMO to be directly utilised in beer.[69]

S. cerevisiae is a model eukaryotic system used in large-scale operations, with a well-studied molecular and cellular biology as well as a variety of genetic tools. As an eukaryotic organism, it contains several organelles that may serve as compartments for the production of different compounds. Its ability to functionally express plant metabolic enzymes is well-known, enabling its common use for polyphenols production, either academically or in an industrial context. [25] To attain microbial polyphenolic compound production, plant-native enzymes are initially overexpressed via either codon optimisation or the construction of enzyme synergies that facilitate bacterial expression.[70] It has a high pH tolerance, which

minimises the likelihood of bacterial contamination and eliminates the need to neutralise acidic substances.[25] An important trait of *S. cerevisiae* strains utilised in hemicellulose-based processes is tolerance to lignocellulose inhibitors, as it can be employed to reduce the inhibitory effect of the presence of acetic acid, furfural, or HMF, especially when considering robust industrial strains, more tolerant to stressors such as the presence of toxic compounds.[71–73] The better fermentation performance of industrial isolates compared to laboratory strains in very high-gravity conditions was related to an increased accumulated content of sterols, glycogen, and trehalose in the industrial isolates.[72]

Due to the higher ideal temperatures of hydrolytic enzymes (50 ºC) compared to the optimal temperature of 30 ºC for *S. cerevisiae* fermentation, thermotolerance is also one of the properties exhibited by some industrial yeast strains that might be advantageous for lignocellulose valorisation, and bioethanol fermentation is the most widely process studied and implemented on a commercial scale context.[18,19,74,75] Industrial isolates may also possess the inherent capabilities and specificities to respond to genetic engineering, either for tolerance or pentose metabolism, highlighting the need for personalised genetic engineering of the yeast chassis and lignocellulosic biomass used in the fermentation process.[15,19,31] These properties have led to the establishment of *S. cerevisiae* as a chassis microorganism for the valorisation of lignocellulosic biomass.[76] Nevertheless, *S. cerevisiae* is incapable of metabolising carbon sources like xylose and lactose, highly available in different agro-industrial wastes, and this is where the wide availability of genetic tools for metabolic engineering strategies comes into play.

1.2.1. Metabolic engineering strategies for the utilisation of non-naturally metabolised carbon sources

1.2.1.1. Lactose

S. cerevisiae cannot natively metabolise lactose due to lacking the necessary transport (lactose permease) and hydrolysis enzymes (β-galactosidase),[49] but it can transport galactose (by the permease encoded by the gene *GAL2*), that is directed to Leloir pathway inside the cell.[77] Nevertheless, its abovementioned advantages for biotechnological applications justify the attempts to circumvent this hindrance.

One of the most attractive characteristics of *S. cerevisiae* is its high osmotolerance, which enables the fermentation of high lactose concentrations (up to 200 g/L).[78] The construction of a lactose-consuming strain has been exploited mainly by employing two different strategies: the construction of recombinant *S. cerevisiae* strains able to produce and secret β-galactosidase to the external medium (Figure **1.2A**) and the construction of recombinant *S. cerevisiae* strains for lactose assimilation and further fermentation

(Figure **1.2B**). In the first, the production of an extracellular β-galactosidase enables its recovery from the broth after fermentation, facilitating the downstream processing. Due to the high complexity and cost of this hydrolase production, coupling its production with other processes like ethanol fermentation constitutes an advantage.[79,80] The other approach consists of the introduction of heterologous genes for lactose assimilation into the cytoplasm and its hydrolysis inside the cells. This ability involves two proteins: lactose permease (encoded by *LAC12* gene) and a β-galactosidase (encoded by *LAC4* gene). The transporter is inducible by intracellular levels of lactose and galactose. It is also an active transporter system that requires energy and can transport lactose against the concentration gradient.[81] The β-galactosidase, also known as lactase, is a cytosolic protein that hydrolyses lactose into glucose and galactose. These two monosaccharides are easily metabolised through glycolysis and the Leloir pathway, respectively.

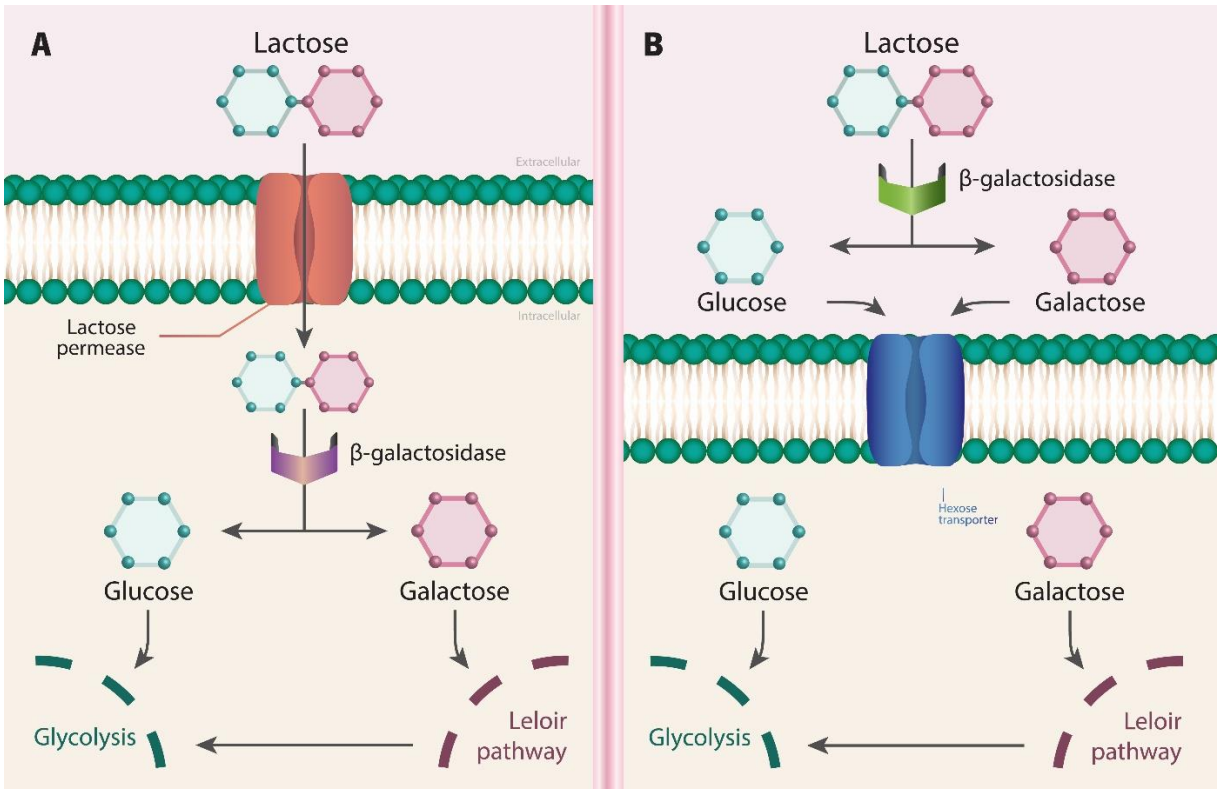

Figure 1.2. Pathways for lactose-consuming *S. cerevisiae* strains. **A.** A heterologous β-galactosidase produced by *S. cerevisiae* is secreted to the external medium, where it hydrolyses lactose into glucose and galactose. The simple sugars are then transported into the cells and metabolised; **B.** A strain expressing a lactose permease which mediates lactose transport into the cell, where it is hydrolysed into glucose and galactose by the action of a heterologous expressed β-galactosidase.

The heterologous expression of the lactose metabolic pathway enables the use of cheese whey as a substrate for *S. cerevisiae*-based fermentation processes. Several studies have reported the use of recombinant lactose-consuming *S. cerevisiae* strains, and while ethanol is the most prevalent product from cheese whey,[82–84] several other compounds of interest have been produced for cheese whey valorisation processes, such as organic acid like lactic acid,[85], prebiotics like tagatose[86] and lactulose,[87] or aromatic compound such as β-carotene.[88] Overall, the yeast *S. cerevisiae* shows itself as a suitable host for engineering lactose metabolising traits.

1.2.1.2. Xylose

Several xylose-assimilating yeasts have been isolated from different environments, but only a small percentage is capable of fermenting this pentose sugar.[89] These naturally xylose-fermenting yeasts, such as *Scheffersomyces stipitis* (formerly known as *Pichia stipitis*), *Candida tropicalis* or *Spathaspora passalidarum* can convert xylose into ethanol, however low tolerance to ethanol and lignocellulosic-derived inhibitors are major limitations, as well as severe culture conditions requirements like pH and dissolved oxygen levels to uphold the xylose fermentation performance.[90]

Given this, a lot of work has been developed over the past few decades to develop *S. cerevisiae* strains that are capable of consuming xylose. This has been accomplished through the expression of xylose assimilation pathways and the optimisation of internal metabolism through metabolic engineering, putting the naturally occurring fermentative capacity of *S. cerevisiae* to use. The most commonly applied strategies are the heterologous expression of the oxidoreductase and the isomerase pathway, with both pathways converting xylose into xylulose (Figure **1.3**).

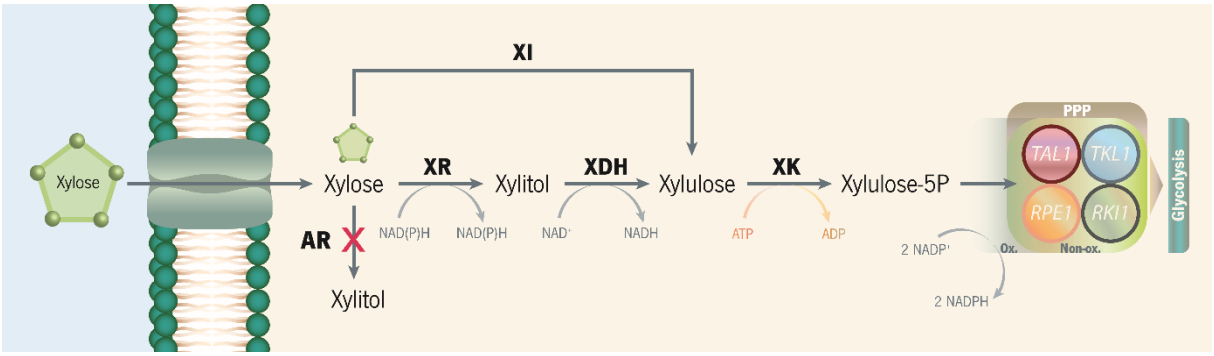

Figure 1.3. Representation of the oxidoreductase and isomerase pathways, the two most used for the development of xylose-consuming *S. cerevisiae* strains. XI, xylose isomerase; XR, xylose reductase. XDH, xylitol dehydrogenase; XK: xylulokinase, AR, unspecific aldose reductase; *TAL1, TKL1, RPE1, RKI1,* genes encoding the enzymes of the non-oxidative (Non-ox.) Pentose Phosphate Pathway (PPP). Ox., oxidative phase of the PPP, where the regeneration of co-factors occurs.

The first is utilised by xylose-fermenting yeasts and occurs predominantly under aerobic conditions. It consists of two enzymatic reactions catalysed by xylose reductase (XR) and xylitol dehydrogenase (XDH), converting xylose to xylulose via xylitol in a two-step redox reaction.[91] This pathway starts with the reduction of xylose into xylitol by XR that preferably uses NADPH as a cofactor, followed by xylitol conversion into xylulose by XDH, in a reaction that only uses NAD^+ as a cofactor.[92] On the other hand, in the isomerase pathway the conversion of xylose into xylulose is a one-step reaction catalysed by xylose isomerase (XI), a reaction without cofactor requirement.[92,93] The majority of XI found in nature has come from bacterial strains, but some anaerobic fungi can also use XI to assimilate xylose.[94,95]

Common to both pathways is the phosphorylation of xylulose into xylulose-5-phosphate by the endogenous xylulokinase (XK), which often requires the overexpression of its coding gene to achieve efficient xylose consumption.[90] Xylulose-5-phosphate is further metabolised through the pentose phosphate pathway (PPP). The first attempts of cloning XI into *S. cerevisiae* failed due to difficulties in expressing functionally-bacterial XI in yeast.[96,97] However, the discovery of XI coding genes from anaerobic fungi[94,95,98] and bacteria[99–101] enabled the successful expression of functional XI and consequent xylose fermentation in *S. cerevisiae*. Additionally, the simultaneous expression of both oxidoreductase and isomerase pathways has shown benefits for xylose metabolism, especially in the non-detoxified hemicellulosic hydrolysate.[102]

The deletion of *GRE3*, a gene that encodes for an unspecific aldose reductase involved in the generation of xylitol, increased xylose assimilation by *S. cerevisiae* expressing bacterial XI because xylitol production was reduced, which inhibited XI activity.[103]. Furthermore, the non-oxidative phase of the PPP is the catabolic route for xylose in fermenting yeasts capable of naturally consuming this pentose sugar, and for xylulose in *S. cerevisiae*.[104] Four enzymes are responsible for the non-oxidative PPP, namely transaldolase (encoded by *TAL1*), transketolase (*TKL1*), D-ribulose-5-phosphate 3-epimerase (*RPE1*) and ribose-5-phosphate ketol-isomerase (*RKI1*), and its overexpression for improved ethanol fermentation and xylose consumption in xylose-only media is broadly reported in the literature.[105–108]

S. cerevisiae does not possess specific xylose transports, using native hexose transporters to assimilate xylose.[109,110] These nonspecific transporters have less affinity to xylose than glucose and show inefficient transport when xylose is present at lower concentrations, constituting a bottleneck in the development of an efficient xylose-fermenting yeast.[111] Therefore, the heterologous expression sugar transporters from native fermenting yeasts, such as *GXF1* from *Candida intermedia*, or *SUT1* from *S. stipitis* have been a successful strategy to improve xylose transport in *S. cerevisiae*, leading to significantly

increased affinity and transport rates of xylose.[112] Altogether, these strategies contribute to the efficient utilisation of *S. cerevisiae* for xylose-based bioprocesses.

1.2.2. CRISPR/Cas9 system

S. cerevisiae is one of the most exhaustively used model organisms for metabolic engineering strategies. In *S. cerevisiae*, many studies have harnessed the native homologous recombination (HR) machinery as a preferred error-free DNA repair mechanism for genomic manipulation.[113] Even though robust HR-based integration of exogenous DNA is efficient in yeast, it requires the use of selection markers for validating and maintaining DNA integration, which has often limited the numbers of edits that can be introduced even in auxotrophic laboratory strains. Similarly, there is limited availability of dominant markers, requiring extensive recycling of them when targeting multiple gene integration.[114]

In the early 2000s, spacer sequences from Clustered Regulatory Interspaced Short Palindromic Repeats (CRISPR) loci from bacteria were found to match viral or conjugative plasmid sequences. This allowed hypothesising that CRISPR should be part of the bacterial immune system.[115] In 2012, two research groups reported that they were able to reprogram the targeting of a CRISPR-associated protein (Cas9), allowing the *in vitro* introduction of sequence-specific double-strand breaks (DSB), which has transfigured the field of genome engineering.[116,117] CRISPR/Cas9 genome engineering has proven to be a fast, marker-free, versatile and, most importantly, targeted genome-editing technique.[118] This allows metabolic engineers to both forward and reverse engineer at much greater efficiencies than earlier.

Briefly, in the CRISPR/Cas9 system, Cas9 is led to a specific target DNA region by a hybrid two-RNA molecule comprising a CRISPR RNA (crRNA) and transactivating crRNA (tracrRNA) and, together, they form a secondary structure loop, which then recruits Cas9.[119] Subsequently, the Cas protein complex is guided by the hybrid crRNA–tracrRNA to a genomic target matching the 20 base pairs from crRNA, where the HNH nuclease domain of Cas9 cleaves the DNA strand complementary to the crRNA-guide sequence, while RuvC-like domain cuts the other strand, therefore resulting in a DSB.[117] The specific genomic target must be tailed by the protospacer adjacent motif (PAM), which has an NGG sequence, where N can be any nucleotide,[120] with the DNA cleavage occurring three nucleotides upstream of the PAM site.[116] Lastly, homologous recombination of donor DNA leads to the generation of a recombinant yeast strain (Figure **1.4**).

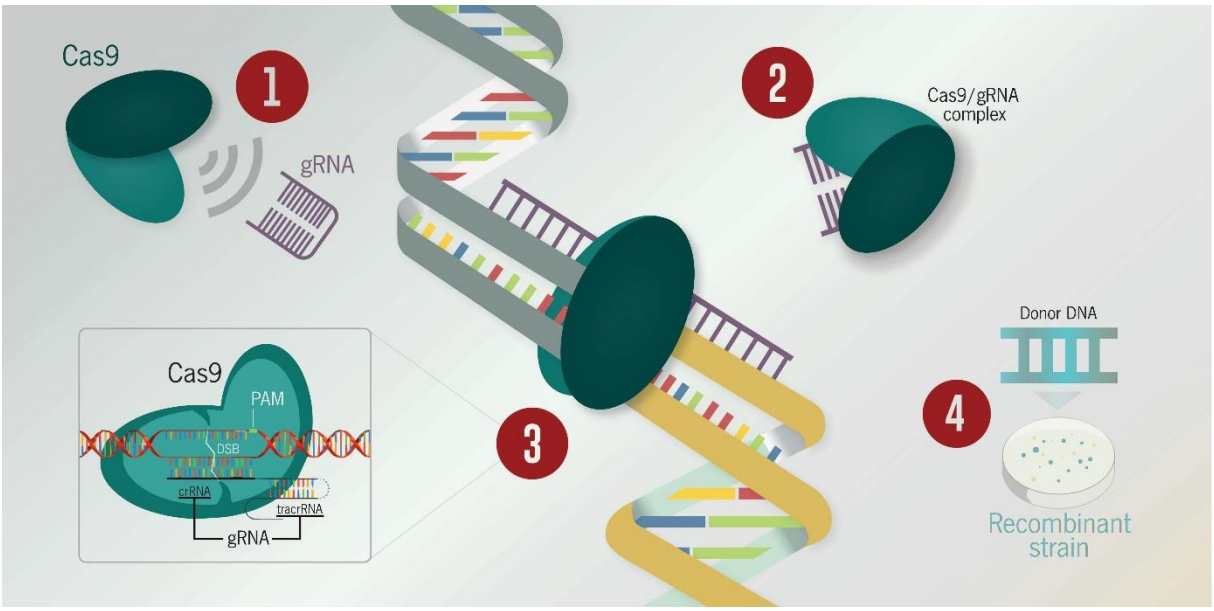

Figure 1.4. Overview of a CRISPR/Cas9-mediated gene integration. (1) Cas9 recruits the gRNA molecule and (2) both form a complex. (3) The Cas9/gRNA complex target the desired insertion site (20 bp) near a protospacer adjacent motif (PAM) site and induce a double-strand break (DSB). (4) After homologous recombination of a donor DNA, a recombinant yeast strain is obtained.

1.2.2.1. Cas9 and gRNA

CRISPR/Cas9 is a programmable immune mechanism, where the tracrRNA is indispensable for both crRNA processing and targeted Cas9 nuclease activity. To facilitate its use in genome editing, the crRNA and tracrRNA can be fused tail to head via a linker, originating a single guide RNA (gRNA).[117] In addition to allow Cas9 targeting to a specific locus, this has potentiated the utilisation of CRISPR/Cas9 as a two-module tool for efficient genetic engineering. CRISPR/Cas9 adaptation for yeast genome engineering is affected by several parameters in relation to gRNA and Cas9 expression, and different strategies have been adopted for the expression of both.

For Cas9, the most common strategy used so far has been the utilisation of a codon-optimised version of *Streptococcus pyrogenes* Cas9 for yeast under the control of a strong promoter,[121–123] even though the use of weak promoters like *ROX3* has also been reported to minimise the impact of *cas9* expression on yeast growth rate.[124] Regarding gRNA expression, it is of vital importance to assure optimal transcription, as correct folding is crucial for the interaction with Cas9.[125] The first approach to gRNA expression was its expression from the RNA polymerase III small nucleolar RNA (snoRNA) *SNR52* promoter, using the 3'-flanking sequence of the yeast tRNA gene *SUP4* as a terminator. This promoter–terminator combination produced four uracils on the 3'-end[126] and, since the original 3'-end of tracrRNAs consists of four uracils,[127] this approach is suitable for gRNA expression in yeast. It is possible to find a great diversity of studies

recurring successfully to the same gRNA expression strategy abovementioned, being the most widespread approach for gRNA expression.[123,128–131]

From this, it is clear that gRNA and Cas9 expression can be controlled by several means. Although numerous genome-engineering strategies have been reported, evaluation of parameters like the number of genomic edits needed, the genetic background of the host, and ploidy level have to be taken into account when using the CRISPR/Cas9 system.

1.2.2.2. Cas9-mediated genome editing

Conventionally, both gene knock-out and knock-in in yeast have been achieved by inserting donor repair templates consisting of linearised double-stranded DNA flanked by homology regions identical to the target DSB site [132]. With the arrival of CRISPR/Cas9, programmable Cas9 will execute sequence-specific DSB according to the target sequence of the gRNA.

DiCarlo et al.[127] were the first to adopt CRISPR/Cas9 in yeast for gene knock-out by HDR using a double-stranded 90 base pairs (bp) oligonucleotide (dsOligo) as a template. This dsOligo consists of homologous ends to the target site, a stop codon allowing the achievement of perfect recombination frequency (100%) and knock-out of the *CAN1* gene, a plasma membrane arginine permease, in haploid yeast cells, without the inclusion of any selection marker. A different experimental setup that targeted marker-free single and multiple gene knock-out through CRISPR/Cas9 was developed, where *CAN1* gene knock-out was accomplished using 20-bp increments of the template oscillating from 0 to 120-bp tested the finest length of the repair template.[133] In a different work, Jakočiunas et al.[122] applied CRISPR/Cas9 for multiplex gene knock-out seeking the development of strains with improved production of mevalonate. A 90-bp dsOligo was used for homology-directed repair for the knock-out of several genes. Furthermore, the PAM site was replaced by a stop codon and a frame shift mutation to hamper constant Cas9 targeting and accumulation of the transcript of the gene, respectively. This resulted in engineering efficiency between 50 and 100% for all edits, with best-performing strains exhibiting up to 41-fold increased mevalonate titres when compared to wild-type levels.[122] Additionally, in diploid industrial strains, CRISPR/Cas9 enabled the disruption of two alleles of the *ADE2* gene in several unrelated strains, with efficiency up to 78%.[123]

In order to produce greener chemicals, based on biotechnological production, the integration of heterologous genes is often needed. With the application of CRISPR/Cas9, marker-free integration of multiple genes, or even full metabolic pathways, has become possible.[122] The first report showed that the *KanMX* gene cassette, encoding G418 resistance, could be integrated into the *CAN1* locus with almost

100% efficiency.[127] Since then, several other studies showed successful Cas9-mediated gene integration, as CRISPR/Cas9 became the main toolkit for genome editing. The powerful combination of HR combined with CRISPR/Cas9 enables multi-loci integration of *in vivo* assembled DNA parts, and this method (CasEMBLR) was successfully implemented for the insertion of the three carotene biosynthesis genes, *crtYB*, *crtI* and *crtE,* into target loci *ADE2*, *HIS3* and *URA3*.[122] Other system configurations can also expand the applications of the system. The Di-CRISPR (delta integration CRISPR-Cas) platform was developed for multi-copy integration of metabolic pathways in delta sites,[134] where a combined 24 kb xylose utilisation and (R,R)-2,3-butanediol (BDO) production pathway was integrated into up to 18 copies and allowed BDO production directly from xylose. CRISPR-based interference (CRISPRi)[135] is also growing in interest. CRISPRi is based on the discovery of two point-mutations in Cas9, in the HNH nuclease domain and the RuvC-like domain, that retract Cas9 endonuclease activity, but keep its gRNA binding activity intact and, this way, still able to specifically target DNA sequences.[117] Not long after this finding, an RNA-guided nuclease-deficient Cas9 (dCas9) was able to support a physical block of transcription initiation and elongation, thus mediating the application of CRISPRi, which can be a valuable alternative to conventional techniques like the use of RNA interference to control the gene expression.[136]

The CRISPR/Cas9 technology allows for fast strain engineering of both prototrophic wild and industrial yeast strains. Also, multiple simultaneous genome edits can be achieved and can be independent of marker cassette integration. For transcriptional regulation, the CRISPR/Cas9 system offers the advantage of an easy design and implementation, the possibility of multiplexing and orthogonality. Nevertheless, some limitations are yet to be surpassed in order to enable wider implementation of CRISPR systems, such as the design of effective and specific targeting for diverse yeast species, abolition of cloning necessity or the ability for large-scale multiplexing.[119]

1.2.2.3. EasyClone-MarkerFree vector set

The EasyClone-MarkerFree vector set was designed for *S. cerevisiae*, taking advantage of the CRISPR/Cas9 system's ability to introduce DSB in the DNA of specific integration sites. This leads to highly efficient integration of expression vectors, bypassing the requirement for selection, originating scarless marker-free yeast strains.[137] This expression system is based on previous versions of this method, which used auxotrophic[138] or dominant[139] selection markers, and comprises the use of a backbone with a bacterial replication origin for plasmid amplification in *Escherichia coli* and a USER (Uracil-Specific Excision Reagent)[140] cloning cassette that enables the insertion of biobricks. Also, two terminators are found in opposite directions, flanking the USER cloning site, and homologous sequences for HR into the

desired integration sites in the genome. These integration sites are set in the intergenic regions of the chromosomes X, XI and XII and are interspaced by essential genes, which helps to guarantee that the integrated DNA fragments are not at risk of removal by HR. These integration sites were previously found to ensure a high level of expression and do not hinder cellular growth.[141]

After yeast transformation is complete, the gRNA helper vectors and/or the Cas9 plasmid can be easily removed by cultivating the recombinant strains on a non-selective medium, and the strain is then prepared for a subsequent transformation. This reduces turnaround time in comparison to alternative methods like the Cre-LoxP-mediated recombination and also eliminates the genome instability associated with marker removal of Cre-LoxP systems,[114] enabling fast and efficient yeast genome editing.

1.3. Plant-derived polyphenols

Aromatic natural products are a class of chemicals with great value for the industry. These occur in nature as plant secondary metabolites, which despite not having a direct influence on plant growth, development, or reproduction, are of major importance to several plant functions, such as defence and signalling, or act as pigments or fragrances. Contrasting to primary metabolites, plant secondary metabolites can differ from species to species and encompass a diverse collection of complex chemical structures. Some of these natural aromatic compounds can serve as the building blocks (e.g. cinnamic acid, *p*-coumaric acid) to produce a wide range of polymers, esters, fibres, and pharmaceuticals and nutraceuticals.[142–144]

Inside this class of aromatic compounds, phenylpropanoids and their derivatives are a wide-range group of phenylalanine- and tyrosine-derived metabolites (C6–C3).[145] Phenylpropanoids are found throughout the plant kingdom, where they serve as essential components of a number of structural polymers, provide protection from ultraviolet light, defend against herbivores and pathogens, and mediate plant-pollinator interactions as floral pigments and scent compounds.[146] As for the majority of aromatic natural products, phenylalanine and tyrosine are initially forked from the shikimate pathway (Figure **1.5**), which only occurs naturally in plants, bacteria and fungi.[147] Chorismate plays a key role in the biosynthesis of several value-added aromatic compounds, such as hydroxycinnamic acids, flavonoids and stilbenoids.[148,149] The enzymes involved in the shikimate pathway have been broadly studied and characterised. The energetic expenditure of synthesising aromatic acids requires twice the ATP cost than most amino acids.[150] Hence, it is expected to encounter severe regulation at the transcriptional and allosteric levels throughout the shikimate pathway.[70]

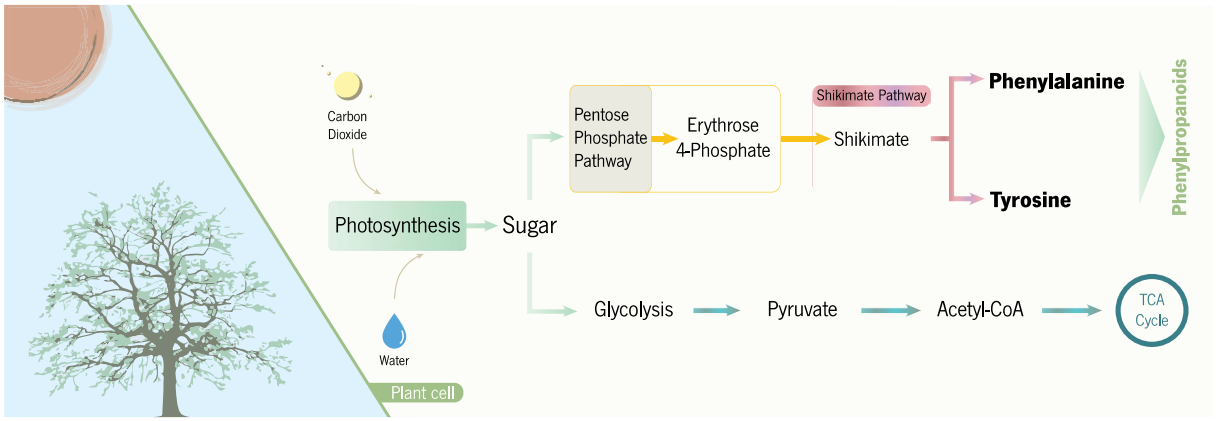

Figure 1.5. Overview of the key steps for the production of tyrosine and phenylalanine, amino acid precursors of the phenylpropanoid pathway, through the shikimate pathway in plants.

1.3.1. Hydroxycinnamic acids

Hydroxycinnamic acids are phenolic compounds with organic carboxylic acid function, with distinct significance due to their biological features and potential applications. The biosynthesis initiates with the conversion of phenylalanine or tyrosine into the building blocks for several types of phenolic compounds. Phenylalanine ammonia lyase (PAL) catalyses the deamination of L-phenylalanine into cinnamic acid, which is then converted to *p*-coumaric acid, a key building block for a wide range of phenylpropanoid derivatives, through the action of cinnamate 4-hydroxylase (C4H). Alternatively, TAL can convert tyrosine directly into *p*-coumaric acid.[151] Common hydroxycinnamic acids are *p*-coumaric acid, caffeic acid, ferulic acid, 5-hydroxyferulic acid and sinapic acid (Figure **1.6**). Furthermore, these phenolic acids are used as gateway intermediates for the biological generation of flavonoids and stilbenoids, among others.[152]

1.3.2. Flavonoids

Flavonoids are a far-reaching class of plant secondary metabolites, conferring protection from pathogens, UV-light and oxidative damage to plants.[153] They are mainly found in the skins and seeds of the grapevine fruit. Flavonoids have a skeleton with two benzene rings combined with a heterocyclic pyrane ring. Several health benefits are associated with them, such as antioxidant, anti-bacterial, anti-carcinogenic and anti-inflammatory properties.[154] Flavonoids can be gathered into numerous sub-groups like flavones, flavonols, flavan-3-ols, dihydroflavanols and anthocyanidins, which have identical skeletons but different oxidation states of their central pyran ring (Figure **1.6**).[155,156]

Flavonoids are produced from a phenylpropanoid-derived coenzyme A (CoA) thioester by the action of chalcone synthases, catalysing chalcone formation. Subsequently, chalcone isomerases catalyse the isomerisation of chalcone into flavanones. From here, flavanone products are originated, which include naringenin, genistein, flavonols, and anthocyanins, among others.[157]

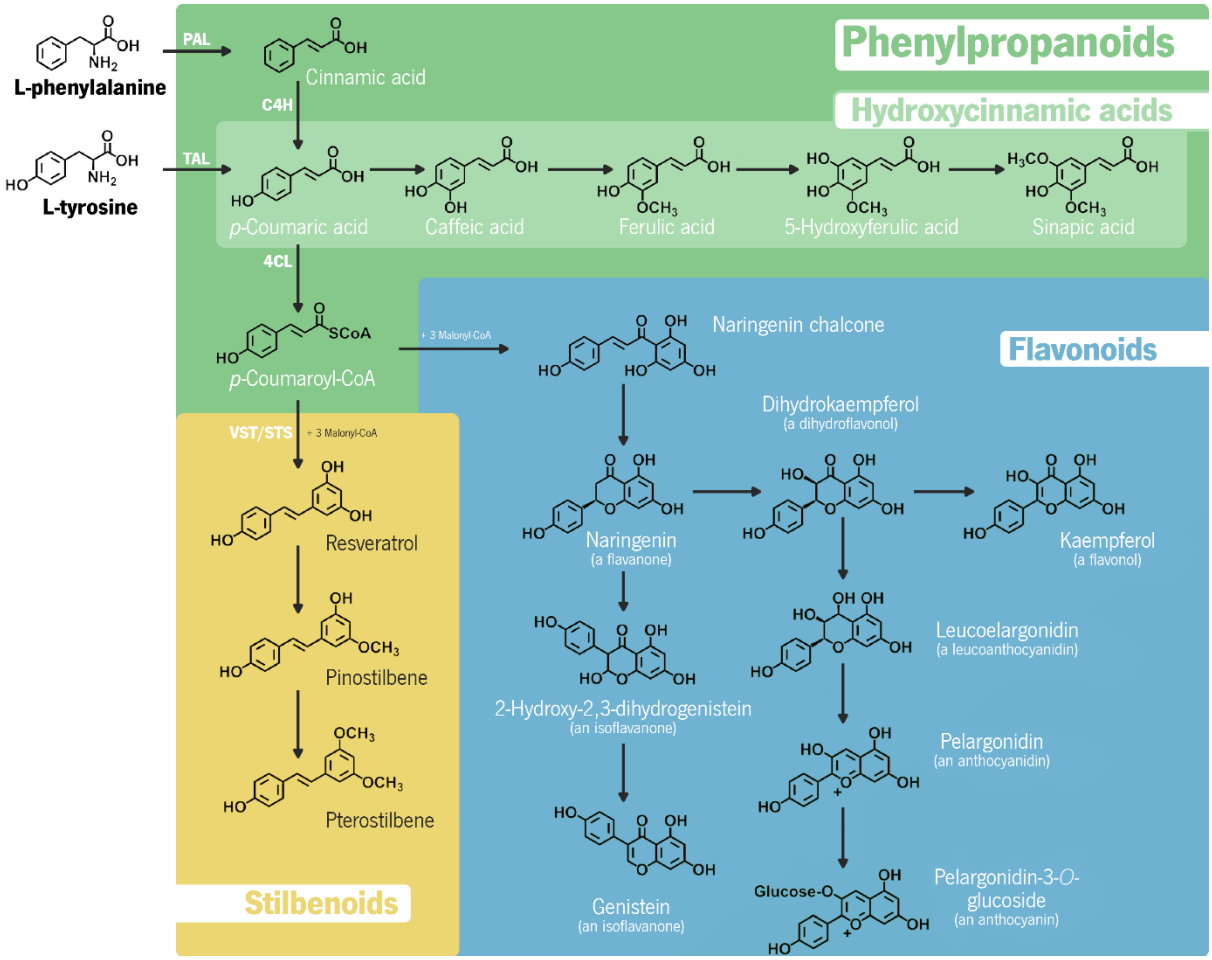

Figure 1.6. Pathways for phenolic acids, stilbenoid and flavonoid synthesis from phenylalanine or tyrosine. PAL, phenylalanine ammonia lyase; TAL, tyrosine ammonia lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate:CoA ligase; STS, stilbene synthase; VST, resveratrol synthase. Adapted from Milke et al.[152]

1.3.3. Stilbenoids

Stilbenoids are another class of phenolic compounds (non-flavonoids) belonging to the phenylpropanoid group and in the last few years have generated great interest because of their potential health valuable effects.[158] Stilbenoids are based upon a 14-carbon skeleton comprising one or two

hydroxylated aromatic rings linked with a methylene bridge. Stilbenoids are plant-derived aromatic natural products with noteworthy biological properties and exhibit significant potential in therapeutic or preventive applications in pharmaceuticals.[159] The biosynthetic pathways for stilbenoids production are strictly related to flavonoids, but instead of CHS and CHI, stilbene synthases are responsible for condensation of CoA-linked cinnamic acids with three molecules of malonyl-CoA to form stilbenoids. Resveratrol, due to its strong antioxidant activity and nutraceutical properties,[160] is the most studied stilbenoid, and its methylation leads to the formation of its derivatives, pinostilbene and pterostilbene (Figure **1.6**).

1.4. Resveratrol

1.4.1. History

Resveratrol (3,4',5-trihydroxystilbene) is a stilbenoid with strong antioxidant activity that plays a role in plant defence against environmental stresses.[161] It was first isolated in 1939 from *Veratrum grandiflorum*, a poisonous plant used at that time for therapeutic applications.[162] The generic name "resveratrol" is speculated to arise from the combination of its chemical structure and the plant source used for isolation: a derivative from resorcinol occurring in *Veratrum* species, comprising hydroxy groups that form alcohol.[163] A couple of decades later, resveratrol was found in root extracts of the Japanese knotweed, *Fallopia japonica* (also known as *Polygonum cuspidatum*),[164] a Chinese medicinal plant considered an invasive species,[165] which is currently the main source of commercialised resveratrol[166]. It was later isolated from the grapes of *V. vinifera* and classified as a phytoalexin, due to its production in plants being driven by the response to fungal infection or exposure to UV light.[167] After the detection of resveratrol in red wine,[168] the "French Paradox" was coined in 1992, which relied on epidemiological data from the French population and correlated the low incidence of coronary heart diseases with moderate consumption of red wine, despite the significant content of high saturated fat in their diet.[169] Even though the French Paradox concept has evolved over the years, as more detailed information on the negative health impact of heavy ethanol consumption[170,171] and the presence of other phenolic compounds with therapeutic properties like flavonols or phenolic acids,[172] this drove the focus to the understanding of the underlaying mechanisms behind the concept. After the reports of the pleiotropic effect connected to cancer prevention and other disease conditions in the late 1990s,[173] resveratrol started being the subject of extensive research, with thousands of scientific reports arising in the following decades (Figure **1.7**).

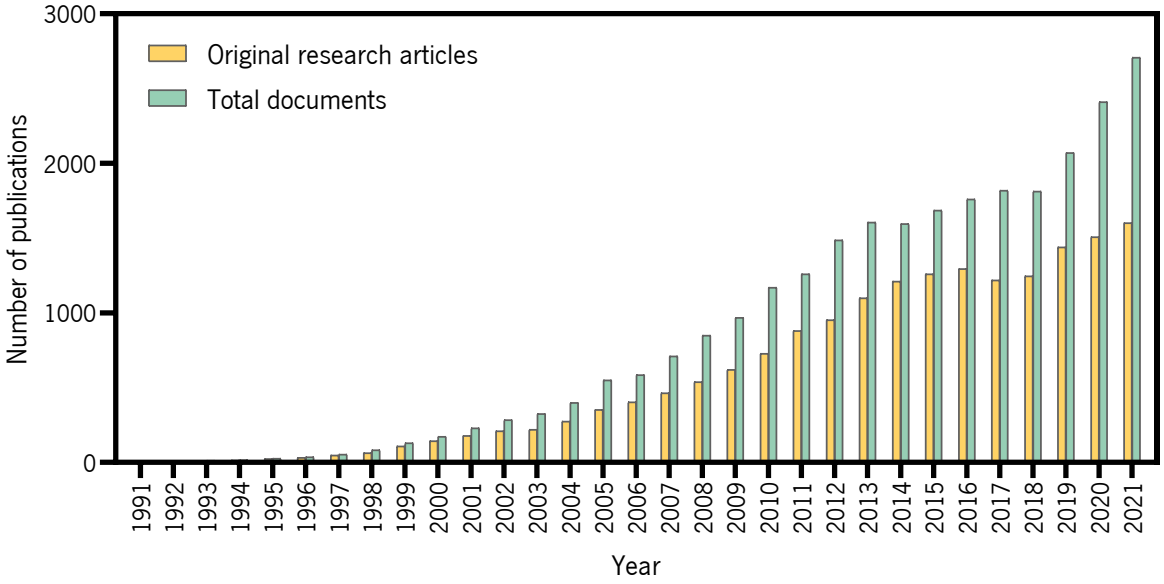

Figure 1.7. Total number of publications contemplating resveratrol in the last 30 years (1991-2021). Data were obtained from Scopus (http://www.scopus.com/) using the queries: TITLE-ABS-KEY (resveratrol) AND (LIMIT-TO (PUBYEAR , YYYY)) for the total number of documents in each year; and TITLE-ABS-KEY (resveratrol) AND (LIMIT-TO (PUBYEAR , YYYY)) AND (LIMIT-TO (DOCTYPE , "ar")) for Original Research Articles in each year, where YYYY corresponds to each year from 1991 to 2021. Accessed in June 2022.

1.4.2. Chemical properties

Resveratrol ($C_{14}H_{12}O_3$, IUPAC name 5-[(*E*)-2-(4-Hydroxyphenyl)ethen-1-yl]benzene-1,3-diol) has a molecular weight of 228.24 g/mol and has two isomers, *trans*-resveratrol (or (*E*)-resveratrol, according to the *E-Z* system) and *cis*-resveratrol (or (*Z*)-resveratrol) (Figure **1.8**), differing in the conformation of the double hydrogen bond that ligates both phenolic rings. These are connected through an ethylene bridge, encompassing two different groups,4-hydroxystyryl and *m*-hydroquinone, which are responsible for their biological properties, by modulation of molecular targets.[174] Due to its aromatic rings, resveratrol is known to display superior free radical scavenging properties in comparison with other antioxidants like vitamin C or E.[175] Both isoforms of resveratrol can be distinguished by distinct UV spectra, with reported maximum absorbance between 304 nm[176] and 306 nm[177] for *trans*-resveratrol and between 280 nm[178] and 286 nm[179] for *cis*-resveratrol. *Trans*-resveratrol is the primarily biologically-active isoform, being responsible for the majority of the therapeutic properties of resveratrol.[180]

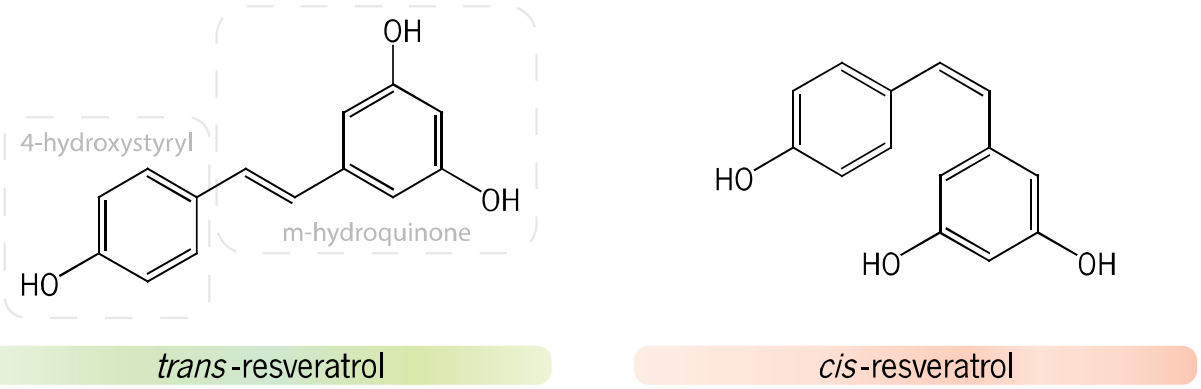

Figure 1.8. Chemical structures of *trans*-resveratrol, with identification of both moieties of the molecule, and *cis*-resveratrol.

1.4.2.1. Stability

Resveratrol is highly reactive to light, pH, and temperature changes, mainly due to its unstable hydroxyl groups and C—C double bond.[181] The most common and stable isoform in nature is *trans*-resveratrol, having higher bioavailability,[182] though it can suffer *cis*-isomerisation under exposure to sunlight[183] or natural and artificial UV radiation[179]. Nevertheless, *trans*-resveratrol can maintain its stability for several months when in the absence of light and a low pH environment.[184] Resveratrol was found to keep its stability at low pH conditions for several days at 37 ºC. After 7 days of incubation, approx. 90% of resveratrol was still preserved at pH 4 or less, and more than 70% at pH 5 or 6.[185] Still, cultivation conditions at 35 ºC were previously found to cause some degradation of resveratrol,[186] so this phenomenon can be also correlated with the incubation temperature and not exclusively dependent on pH. At pH 8 or above, less than 2% of resveratrol remained intact, showing its clear instability in basic conditions.[185]

1.4.2.2. Solubility

Resveratrol has relatively high lipophilicity (log P = 3.4),[187] being practically insoluble in water,[188] which hinders severely its bioavailability. It is widely reported to be soluble in ethanol,[185,189], and slightly soluble in natural food-grade oils, like soybean, almond or olive oils,[190] which has additional interest due to its possible incorporation into foods with constant oil phase, like butter or cooking oils.[191] Resveratrol is also very soluble in surfactants/detergents, like Tween 20,[189] and the non-ionic emulsifier polyethylene glycol 400 (PEG 400), with a reported solubility up to 374 g/L.[185] Recent studies using natural deep eutectic solvents (DES), a non-toxic and highly recyclable class of ionic liquids,[192] also showed their potential as

solvents for resveratrol, with DES like Choline chloride:glycerol, at a molar ration of 1:2, exhibiting a solubility of 321 g/L.[193] Reported solubility of resveratrol in several solvents is listed in Table **1.1**.

Table 1.1. Resveratrol solubility in different solvents.

Compound	Solubility (g/L)	Temperature (ºC)	Ref.
Water	0.03-0.05	Room	188,194
Ethanol	87.98 – 129.86	Room - 37 ºC	185,189
Glycerine	3.83	37 ºC	189
Olive oil	5.23	Room	190
Soybean oil	4.004	Room	190
Almond oil	4.066	Room	190
Tween 20	107.75 – 248.38	37 ºC	189,195
Tween 60	105.16	37 ºC	189
Tween 80	7.46 – 76.8	Room - 37 ºC	185,190,195
PEG 400	139.95 – 373.85	Room	185,189
DES 1,2-propanediol:choline chloride:water (1:1:1)	> 20	40 ºC	196
DES Choline chloride:glycerol (1:2)	321	n.r.	193

n.r. not reported

1.4.3. Biological activities and therapeutic applications

The 21st century has been particularly prolific in an extensive study of the biological activities of resveratrol and its application as a therapeutic agent. These activities are mainly based on the ability of resveratrol to modulate multiple cell signalling molecules like sirtuin type 1 (SIRT-1), highly associated with ageing,[197] insulin-like growth factor 1 (IGF-1), insulin-like growth factor-binding protein 3 (IGFB-3), Ras association domain family 1α (RASSF-1α), cytokines, caspases, among many others, therefore being considered a multitargeting agent.[198] As long-term use of rationally designed medications is frequently associated with many adverse effects, the shift to multitargeted therapies, that are less potent but still modify several targets to elicit multiple responses, is desired.[198] Resveratrol has been recognised as having multiple bioactive properties, such as antioxidant, cardioprotective, anti-inflammatory, anti-ageing, neuroprotective, and anticancer activities, among others.[199]

Resveratrol is rapidly absorbed and metabolised in the human body, and low bioavailability (roughly 1% when taken orally) are some of the main limitations to its therapeutic use.[200] This is particularly challenging when addressing organs that are far from the digestive tract,[201] but clinical research has shown that resveratrol is effective on its own or in conjunction with other medications,[202] and efforts are being made to side this restraint. Micronised resveratrol (SRT501) has been reported to have 3.6-fold higher levels in plasma (1942 ng/mL) from a single dose administration when compared to standard resveratrol,[203] and its concentration in hepatic tissues was equivalent to pharmacologically effective doses shown in preclinical tests.[204] Efforts have also been made in the field of encapsulation of resveratrol, protecting its functionality and viability,[205] and many materials such as whey proteins have been investigated as carrier material for encapsulation of bioactive compounds,[206,207] including resveratrol.[208] Resveratrol has been taken in the quotidian diet for a long time, being therefore well-tested from a safety standpoint. It was found to be safe in doses up to 5 g per day.[209] Certain side effects were observed in a relatively small number of patients, consisting mainly of mild and sporadic conditions like headache, dizziness and epididymitis, by recurrent administration in short periods of time or high doses (2.5 and 5 g/day).[210]

Even though the pleiotropic activities of resveratrol have been explored for a long time,[173,211] the vast part of the discussion on these effects relied primarily on preclinical tests. Currently, there are more than 250 clinical trials either complete or ongoing focusing on resveratrol effects on human health, as the sole therapeutic agent or combined with other pharmacological compounds, with more than 95% being developed in the last decade.[198] Some of the outcomes of clinical trials completed so far are discussed below.

1.4.3.1. Ageing

Resveratrol as an anti-ageing agent is one of its most prominent properties since the first studies on its ability to mimic calorie restriction effects and expand the lifespan of several (micro)organisms by activating the sirtuin pathways were reported.[197,212] Sirtuins are regulator proteins and their overexpression is associated with extended longevity, and resveratrol is known to indirectly activate sirtuin 1 (SIRT1) in humans.[211–213] Although being still advertised as an antiaging combination, based on scientific literature reports with mechanistic foundations, there is still no definitive proof that resveratrol has a direct impact on increasing the lifespan of humans or other organisms.

Nevertheless, one of the major commercial applications of resveratrol is in facial skin products. A resveratrol-based cosmetic product displayed 17 times the antioxidant capacity of idebenone, a

commercial drug with effective antioxidant effects, protecting against cell damage from oxidative stress, which topical application prevented photoaging.[214] Other known beneficial effects of its topical use include wrinkle reduction and enhanced skin vitality,[215] anti-scarring,[216] acne reduction,[217] and wound healing properties,[218] supporting its wide utilisation, especially in anti-ageing facial skin products. Additionally, distinct nanocarriers for topical administration and microemulsions (e.g. containing a large amount of natural deep eutectic solvents)[219] can also improve resveratrol solubility, provide photoprotection, enhance skin penetration and inhibit *trans*-to-*cis* isomerisation.[220]

1.4.3.2. Cancer

In malignant hepatic tissue of patients with colorectal cancer, the micronised resveratrol SRT501 considerably boosted the expression of cleaved caspase-3, which might be linked with improvement in the disease.[203] In patients with colon cancer, downregulation of the antigen CD133 (prominin-1) and LGR5 (leucine-rich repeat-containing G-protein coupled receptor 5) was observed, which are deeply connected with growth inhibition of colon cancer cells.[221] In multiple myeloma patients, though, resveratrol administration led to adverse implications.[222] Resveratrol was found to diminish IGF-1 and IGFBP-3 levels in healthy volunteers,[209] of which are associated with different types of cancer, highlighting its potential not only as a treatment but as a cancer chemo preventive agent. Though, additional clinical trials are necessary to support the thousands of reports presenting preclinical tests.

1.4.3.3. Diabetes

Resveratrol has demonstrated the ability to combat metabolic disorders such as diabetes mellitus[223] and obesity.[224] Several positive outcomes have been reported regarding the effects of resveratrol in type-2 diabetes. Significant reduction in fasting blood sugar, as well as a reduction in haemoglobin A1c (HbA1c), insulin levels, and insulin resistance was observed.[225] Notably, the significant reduction in HbA1c levels observed in this study was comparable with the reduction in type-2 diabetes patients treated with metformin, a first-line commercial treatment for diabetes.[226] Other trials with patients with type-2 diabetes revealed a reduction in oxidative stress and blood sugar levels and enhanced insulin solubility.[227] Effects on quicker healing and size reduction of foot ulcer[228] and enhanced vasodilator function were also reported.[229] Regarding other metabolic diseases such as obesity, resveratrol administration led to significant loss of weight in obese patients after 6 months of treatment.[230] Significant reduction in oxidative stress,[231] improved adipogenesis and a decrease in adipocyte size were also reported.[232] The use of resveratrol alone or as an adjuvant has been established in various clinical studies, with very encouraging

results in the field of diabetes treatment and other metabolic diseases. Still, there is a lot to learn about the best resveratrol dosages for auxiliary treatment.

1.4.3.4. Cardiovascular diseases

The French Paradox sparked the initial interest for substantial study into the impact of resveratrol on cardiovascular function. Resveratrol shows up as a promising treatment for cardiovascular disease (CVD), the principal cause of mortality worldwide, but only a few clinical trials have been carried out in support of these claims.[233] The majority of the clinical trials with significant impact on patients have applied resveratrol combined with other therapeutic agents, and given this, the extent of its influence on the pharmaceutical mixture remains unclear. Some clinical trials reported a considerable decrease in cholesterol and alipoprotein-B, known cardiovascular risk factors, in primary CVD.[234] Increase in blood lipid profile and decrease in fasting blood sugar concentration was also reported in patients with CVD[235] and enhanced diastolic function of the left ventricle and endothelia function was observed in post-myocardial infarction patients, as well as a reduction in low-density lipoprotein (LDL cholesterol).[236] Future clinical trials should address more thoroughly the effect of different factor like ethnicity, age, and gender of the patients, which appears to have an important role in resveratrol effectiveness for CVD treatment.[198]

1.4.3.5. Neurological diseases

Resveratrol has an important role in neuroprotection by lowering oxidative damage, mitochondrial malfunction, and chronic inflammation in the brain and nervous system.[237,238] Research has shown that resveratrol improves memory by strengthening the functional connectivity of the hippocampus, as well as enhancing glucose metabolism, in healthy elder adults (50-80 years old).[239] Even though Alzheimer's disease is one of the most prevalent neurodegenerative illnesses for which resveratrol has demonstrated therapeutic potential, as this disorder has been linked to oxidative stress and mitochondrial damage through multiple genes and mechanisms,[240,241] so far clinical trials on this illness have not reported significant effects to its treatment.[242]

1.4.3.6. COVID-19

The fast-spreading COVID-19, a contagious disease caused by infection by SARS-CoV-2 (severe acute respiratory syndrome coronavirus 2), was first reported in late 2019 in Wuhan (China). This disease, which quickly evolved to a pandemic level, is characterised by pneumonia and acute respiratory distress syndrome (ARDS), leading to the necessity to find urgent solutions for its treatment and containment.[243]

Several publications recently demonstrated the potential application of resveratrol or its derivatives in this field. Resveratrol-derived pterostilbene showed antiviral activity against SARS-CoV-2 in a dose-dependent way, potentially via reducing viral replication.[244] Resveratrol also appears to play a role in the regulation of the renin-angiotensin system and production of angiotensin-converting enzyme 2, modulating the immune system, and downregulating pro-inflammatory cytokines.[245] A meta-analysis study on the current literature concluded that resveratrol might have a potential effect on easing ARDS symptoms by suppressing severe inflammation provoked by SARS-CoV-2, exhibiting significant effectiveness against a variety of DNA and RNA viruses, and regulating molecular pathways by interaction with, such as the tumour necrosis factor TNF-α, interleukin 6 (IL-6) or interleukin 1 beta (IL-1β), among many others, often associated with virus infection.[246] Further elucidation on these effects should be addressed in the near future, and two clinical trials are already in motion to evaluate the anti-fibrotic therapeutic effects of resveratrol for discharged COVID-19 patients and the effect of resveratrol-assisted zinc therapy for reduction of the severity of SARS-CoV-2 viral load.[247]

1.4.4. Routes for resveratrol commercialisation

Currently, resveratrol is mainly commercialised as an ingredient for food and cosmetics and as a dietary supplement, but pharmaceuticals and personal care products are also other end uses for this.[248] As of 2022, the resveratrol market size is nearly €70 million (conversion rates as of May 2022)[249] with an estimated compound annual growth rate (CAGR) of 8.4% for the forecast period of 2021-2031.[248] At the moment, Europe dominates the worldwide resveratrol market, and while plant extraction is still the main source of commercial resveratrol, chemical synthesis (DSM, Netherlands) and microbial fermentation (Evolva, Switzerland) are gaining ground in the market.[166,248]

1.4.4.1. Chemical synthesis

Based on the first report on the isolation of resveratrol by Takaoka in 1939,[162] the first study on the chemical synthesis of resveratrol with profiling of the synthetic substance through comparison with the natural substance came soon after, in 1941, using a Perkins-type condensation reaction.[250] Since then, several different methods have been reported to produce resveratrol. Some of the most commonly used are still Perkins-reaction, Heck-reaction and Wittig-reaction.[181]

Sodium acetate, a base and an acid treatment are used in the Perkin-reaction to transform aromatic aldehydes and anhydrides into α- and β-unsaturated carboxylic acids, involving various steps like protection, condensation, decarboxylation, and deprotection.[251] Several improvements have been

described to this reaction, with resveratrol yields of 85% being reported.[252] Nevertheless, some steps in this reaction require extreme conditions like high temperatures, and a metal catalyst, limiting its broad application.[181] The Heck reaction relies on the C—C coupling reaction of an aryl halide or vinyl halide with an activated olefin in the presence of palladium and a base, with many reports of yields in the 70%-range.[253,254], but preparing the Heck reaction precursor usually requires many stages or expensive catalysts.[255] By its turn, in the Wittig-reaction, primary/secondary alkyl halide and aldehyde/ketone are rearranged to create an olefin product under the action of triphenylphosphine and a base, freeing a triphenylphosphine-oxide by-product,[256] being commonly utilised to form C—C double bonds.[257]. Yields up to 83% have been reported using this type of chemical synthesis[258], but concerns regarding the yield on *trans*-resveratrol have been raised, generating the by-product triphenylphosphine oxide, and requiring chromatographic purification.[181] Its combination with the Heck-reaction, where Wittig-generated styrene was employed in a palladium-catalysed coupling (Heck-reaction) with an appropriate aromatic halide, was previously reported, which resulted in complete *trans*-resveratrol stereoselectivity.[253,254] Other methods for chemical synthesis of resveratrol comprise the Horner–Wadsworth–Emmons reaction[259] and Sonogashira type reaction[255], among many others.

The yields via chemical synthesis are relatively high, however, the chemical synthesis of resveratrol is challenging and contamination and control of the region selectivity and stereo-chemistry of the synthetic process is a major concern.[260,261] The intricacy of the chemical synthesis procedures and the availability of several undesirable by-products restrict the use of chemically-synthesised resveratrol produced, aiding to understand why plant extraction is still the preferred choice for resveratrol. [260]

1.4.4.2. Plant extraction

Resveratrol naturally occurs in more than 70 plant species,[199] most of which are edible, such as grapes (*V. vinifera*),[262] peanuts (*Arachis hypogea*),[263] cocoa (*Theobroma cacao*)[264] and some berries (*Vaccinum* sp.)[265], among many others.[266] Together with the Japanese knotweed, grapes (namely grape skin) and peanuts are the most abundant natural sources of resveratrol.[181,267] Resveratrol biosynthesis, mainly in its *trans*-isomer, in plants, occurs via the phenylpropanoid pathway, using as precursors the aromatic amino acids L-phenylalanine (L-Phe) or L-tyrosine (L-Tyr), synthesised through the shikimate pathway.[268]

To date, several methods for resveratrol extraction from plant material have been reported. These encompass methods like organic solvents extraction, ultrasonic-assisted extraction, enzymatic extraction, pressurised liquid extraction, sequential molecularly imprinted solid-phase extraction, and ionic liquid-based salt-induced liquid-liquid extraction, among many others (Table **1.2**).[269]

Table 1.2. Plant extraction methods of the major species with higher resveratrol content. Adapted from Huang et al.[269]

Species	Extraction method	Yield (mg/g)	Ref.
Vitis vinifera	Pressurised liquid extraction	0.002	270
	Supercritical fluid extraction	7.1	271
	Solid-liquid extraction	4.3	272
Fallopia japonica (*Polygonum cuspidatum*)	Aqueous two-phase extraction	86%*	273
	Ultrasonic-assisted extraction	3.5	274
	Acid hydrolysis, alkali washing	9	275
	Ionic liquid-based salt-induced liquid-liquid extraction	3.9	276
	Macroporous resin adsorption, RP-HPLC	1.4	277
Arachis hypogea	Organic solvent extraction	1.3	278
	Multi-stage counter-current extraction	9	279

* Yield only reported in percentage.

Resveratrol is then usually purified from the extracts by centrifugal partition chromatography,[280] macroporous adsorption resins,[281] high-speed counter-current chromatography,[282] and thin-layer chromatography[283], being primarily detected by HPLC afterwards due to having the lowest detection limit in comparison to other methods like chemiluminescence and/or fluorescence assays.[284]

Transgenic plants and plant cell suspensions have already been considered for enhanced resveratrol production, but even though designed plants may produce up to 650 mg of resveratrol per kg of plant (in fresh weight), the usage of elicitors (chemicals that promote any form of plant defence), extended production durations, purity, and engineering process remain as downsides for employing this technology.[285] Plant cell suspensions also require the use of elicitors to generate a resveratrol concentration up to 5 g/L, and its biggest drawback is its reliance on light, which is not practical for large-scale manufacturing.[286]

1.4.4.3. Microbial production

Resveratrol microbial production by fermentation can side many of the drawbacks associated with the aforementioned alternative non-sustainable nor environmentally friendly routes for its obtainment. Plant extraction of resveratrol is highly dependent on the availability of plant resources, and its geographic diversity is also a concern, relying heavily on environmental factors like weather, climate changes or pest

invasion. Moreover, its extraction process is very complex, lowering the extraction efficiency,[270–272] with high variations in its purity.[275] Alternatively, although enabling relatively high yields, chemical synthesis also poses several disadvantages, such as often requiring multiples steps and harsh conditions (high temperatures, pollutant catalysts, etc.),[287] lack of stereoselectivity, leading to a potential mixture of both isomers,[287] and ease of contamination by different undesirable by-products.[260] Microbial biosynthesis of resveratrol is, therefore, a valuable alternative, posing several advantages, such as reduced costs, obtention of a product with high purity, requires simple purification processes[288] and is independent of season changes[166], being sustainable and environmentally friendly. The primary isomer microbially resveratrol produced is *trans*-resveratrol.

1.4.4.3.1. Engineered *S. cerevisiae* for resveratrol biosynthesis

The first reports on microbial resveratrol production relied on *p*-coumaric acid as a substrate (Table **1.3**). The first study goes back to 2003, when Becker et al.[289] co-expressed the coenzyme-A ligase gene *4CL216* from a hybrid poplar and the grapevine resveratrol synthase gene *VST1*, in a laboratory *S. cerevisiae* strain. This led to a titre of 1.45 µg/L of resveratrol in the form of piceid, a resveratrol glucoside, by feeding 10 mg/L of *p*-coumaric acid to the yeast culture. Soon after, the integration of *4CL2* from *Nicotiana tabacum*, encoding for a 4-coumarate-CoA ligase, and *STS* from *V. vinifera*, encoding for stilbene synthase, achieved a production of 5.8 mg/L of non-glycosylated resveratrol from 820.8 mg/L of *p*-coumaric acid.[288] Expression of *4CL1* from *Arabidopsis thaliana* and *STS* from *A. hypogaea* enabled a titre of 3.1 mg/L of resveratrol from 15.3 mg/L of *p*-coumaric acid.[290] All these studies used laboratory *S. cerevisiae* strains. In its place, Sydor et al.[291] expressed *4CL1* from *A. thaliana* and *STS* from *V. vinifera* into an industrial yeast, isolated from a sugar cane plantation in Brazil. This resulted in the production of 391 mg/L of resveratrol, the highest resveratrol titre at that time, from 2.46 g/L *p*-coumaric acid in YPD medium.

Although the claim for the first report on *de novo* resveratrol production came only a few years later, the first steps toward it came with the attempt to use the amino acid precursors of the phenylpropanoids pathway (Table **1.3**). After a failed first attempt on resveratrol production from tyrosine, due to complications with the expression of tyrosine ammonia lyase (*TAL*) from *Rhodobacter sphaeroides* in *S. cerevisiae*,[292] resveratrol production from this amino acid was accomplished by using a codon-optimised *TAL* gene. Together with the expression of a fused 4-coumaroyl-CoA ligase::stilbene synthase with a Gly-Ser-Gly linker (previously reported to enhance resveratrol yield[292]) and *araE*, encoding for an arabinose-proton symporter from *E. coli*, the strain achieved a titre of 1.90 mg/L of from 12 mg/L of tyrosine on

induction media with SD dropout and 2% (w/v) of galactose.[293] Interestingly, without the addition of tyrosine, 1.06 mg/L resveratrol was accumulated in the fermentation media. In alternative to TAL, phenylalanine ammonia lyase (PAL) from *Rhodosporidium toruloides* was found to have catalytic activity on both tyrosine and phenylalanine,[294] and its introduction in *S. cerevisiae* alongside *AtC4H* (encoding for cinnamic acid 4- hydroxylase) and *AhSTS* led to a resveratrol production of 3.4 mg/L from 2% galactose, accumulating also 2.6 mg/L of *p*-coumaric acid.[295] After switching the *ACC1* promoter for a stronger galactose-inducible *GAL1* promoter (leading to a 2-fold increase in *ACC1* transcription levels) in an attempt to increase the pool of malonyl-CoA, and supplementing the media with 12 mM of tyrosine, a titre of 5.8 mg/L of resveratrol was obtained.[295] The introduction of *PAL* was also attempted to produce resveratrol using phenylalanine as a precursor. By the expression of *PAL* from a *Populus* hybrid, *C4H* and *4CL* from *Glycine max* and *VvSTS*, alongside the expression of a NADPH-cytochrome P450 reductase (CPR) from the same *Populus* hybrid to increase the activity of *C4H*. An accumulation of 0.29 mg/L of resveratrol was observed when the medium was supplemented with 10 mM phenylalanine, and a nearly identical titre of 0.31 mg/L when supplemented with 1 mM *p*-coumaric acid.[296] Vos et al.[297] provided valuable insight on the influence of specific growth rates on the physiology of resveratrol-producing previously engineered yeast strains.[298] Resveratrol production processes require high amounts of energy, with an estimation of 13 moles of ATP per mole of resveratrol being required. Given this and the participation of critical precursors that are growth rate-dependent, a stoichiometric study indicated that the *de novo* generation of resveratrol was closely associated with the yeast cell growth rate. The introduction of the resveratrol pathway into *S. cerevisiae* led to clear transcriptional changes in precursor biosynthesis genes, namely *TKL1*, *ARO7*, and *ARO9*, altogether underlining the need to decouple growth and synthesis of resveratrol for industrial use.[297]

Up to this point, academic studies using *S. cerevisiae* strains were mostly based on proof-of-concept approaches and still exhibited fairly low resveratrol titres (below 10 mg/L). Li et al.[299] reported, for the first time, efficient *de novo* production of resveratrol from a cheap carbon source, such as glucose or ethanol, in a minimal medium, via tyrosine. The authors introduced multiple copies of the genes *TAL* from *Herpetosiphon aurantiacus*, encoding for tyrosine ammonia lyase, *At4CL1* from *A. thaliana* and *VST1* from *V. vinifera*, into a strain overexpressing the feedback-inhibition resistant versions of DAHP synthase (*ARO4*^{K229L}) and chorismate mutase (*ARO4*^{K229L}), and the inactivation-resistant version of acetyl-CoA carboxylase (*ACC1*$^{S659A, S1157A}$). In fed-batch fermentation, the final recombinant haploid laboratory strain (a CEN.PK102-5B derivative), achieved resveratrol titres of 415.65 and 531.41 mg/L from glucose and ethanol feeds, respectively, after an initial batch phase on 40 g/L of glucose in both fermentations. Later

on, the same authors reported resveratrol production from glucose via the phenylalanine pathway by applying a pull-push-block engineering strategy. This comprised multicopy overexpression of the resveratrol biosynthetic pathway (*AtPAL2*, *AtC4H*, *At4CL2* and *VvVST1*), optimisation of the electron transfer to the cytochrome P450 monooxygenase, increase of the precursor supply, and the block of competing pathways to avoid degradation of pathway intermediates.[305] The resultant laboratory recombinant strain produced 812 mg/L of resveratrol in fed-batch fermentation, with a feed of 88 g/L of glucose and a batch phase of another 40 g/L of glucose, which is still the highest resveratrol titre reported in the literature using *S. cerevisiae* (Table **1.3**).[305] Nevertheless, the reported yield, a parameter often overlooked and rarely reported in the vast majority of studies, was still only 0.007 mol/mol glucose, still far from the theoretical maximum of 0.28 mol/mol glucose.[297]

The importance of rewiring the carbon flux towards aromatic amino acid (AAA) biosynthesis for high-level production of its derivates was also shown by extensive engineering of a laboratory *S. cerevisiae* where the authors introduced a phosphoketalose-based pathway to deflect glycolytic flux towards erythrose 4-phosphate formation.[300] By expressing *AtPAL2, At4CL1, ScCYB5, AtATR2, AtC4H* and *VvSTS* alongside several other genetic modifications to optimise carbon distribution amid glycolysis and the AAA biosynthesis, the authors reported production of only 32.1 mg/L of resveratrol, but a high accumulation of *p*-hydroxycinnamic acid, a resveratrol precursor (approx. 2.5 g/L). Further expression of the feedback-insensitive $ACC1^{S659A,S1157A}$, which increased the supply of malonyl-CoA, significantly boosted resveratrol production to 263.4 mg/L, while still accumulating nearly 2 g/L of *p*-hydroxycinnamic acid, that indicates the efficient reroute of the carbon flux.[300] These studies coincide with the upsurge of revolutionary genome-editing technologies like CRISPR/Cas9. Until then, the vast majority of studies focusing on resveratrol production in yeast used expression vectors relying on the presence of auxotrophic markers and galactose induction.[290,292,293,296,301] Modular engineering and co-culture fermentation to divide the metabolic labour between two microorganisms were also assessed for resveratrol bioprocesses. Through constitutive overexpression of *TcTAL*, feedback-inhibition-resistant DAHP synthase (*aroGfbr*) and chorismate mutase/prephenate dehydrogenase (*tyrAfbr*) in a strain lacking *tyrR*, a transcriptional regulator that represses tyrosine synthesis, the upstream *E. coli* module can produce *p*-coumaric acid into the culture medium. The excreted *p*-coumaric acid is subsequently utilised by an *S. cerevisiae* strain, expressing *At4CL, VvSTS* and $ACC1^{S659A,S1157A}$, producing 36 mg/L of resveratrol after optimisation of the inoculation ration between the two populations.[302] Noteworthy, until now, only two other studies reported the use of industrial yeast strains for resveratrol production.[291,303] These studies, though, relied on an episomal plasmid with resistance markers, requiring the presence of antibiotics in the media, which is costly and

unfeasible from an industrial point of view. Additionally, to the best of our knowledge, no efforts have been developed regarding the utilisation of renewable carbon sources like agro-industrial wastes and non-naturally metabolised carbon sources by *S. cerevisiae* until the study where this thesis relies on.

Table 1.3. Studies reporting microbial production of resveratrol using *S. cerevisiae* strains.

Ref.	Year	Relevant genotype	Engineering strategy	Substrate/ precursor fed	Titre (mg/L)
289	2003	*4CL* (*Populus trichocarpa* × *Populus deltoids*) *STS* (*V. vinifera*)	Episomal plasmid	*p*-coumaric acid	$1.45 \cdot 10^3$
288	2006	*4CL* (*N. tabacum*) *STS* (*V. vinifera*)	Genomic integration (single copy)	*p*-coumaric acid	6
292	2006	*4CL*::*STS* (*A. thaliana*, *V. vinifera*) Fusion enzyme	Episomal plasmid	*p*-coumaric acid	5.25
296	2009	*PAL*, *CPR* (*P. trichocarpa* × *P. deltoides*) *C4H*, *4CL* (*Glycine max*) *RS* (*V. vinifera*)	Episomal plasmid	*p*-coumaric acid	0.31
291	2010	*4CL* (*A. thaliana*) *STS* (*V. vinifera*)	Episomal plasmid	*p*-coumaric acid	391
293	2011	*TAL* (*R. sphaeroides*) *4CL*::*STS* (*A. thaliana, V. vinifera*) *araE* (*E. coli*)	Genomic integration (single copy)	*p*-coumaric acid	3.1
290	2011	*4CL1* (*A. thaliana*) *STS* (*A. hypogaea*)	Episomal plasmid	*p*-coumaric acid	3.1
295	2012	*PAL* (*R. toruloides*) *4CL* (*A. thaliana*) *STS* (*A. hypogaea*) *ACC1* (*S. cerevisiae*)	Episomal plasmid	Galactose L-tyrosine	3.4 5.8
301	2012	*4CL1* (*A. thaliana*) *STS* (*V. vinifera*)	Episomal plasmid	*p*-coumaric acid	14.4
304	2015	*4CL* (*A. thaliana*) *STS* (*V. vinifera*)	Episomal plasmid	*p*-coumaric acid	8.249

Table 1.3. (continued)

Ref.	Year	Relevant genotype	Engineering strategy	Substrate/ precursor fed	Titre (mg/L)
299	2015	*TAL* (*H. aurantiacus*) *4CL* (*A. thaliana*) *VST* (*V. vinifera*) *ARO4*^{fbr}, *ARO7*^{fbr}, *ACC1* (*S. cerevisiae*)	Cre-LoxP recombination (single/multi copy)	Glucose and ethanol	531.41
305	2016	*PAL, C4H, 4CL, ATR2* (*A. thaliana*) *VST* (*V. vinifera*) *ACS* (*S. enterica*) *ARO4*^{fbr}, *ARO7*^{fbr}, *CYB5*, *ACC1*$^{S659A, S1157A}$ (*S. cerevisiae*) *ΔARO10*	Cre-LoxP recombination, CRISPR/Cas9-mediated gene integration (single/multi copy)	Glucose	812
300	2019	*PAL2*, *4CL1*, *ATR2*, *C4H* (*A. thaliana*) TAL (*F. johnsoniae)* *STS* (*V. vinifera*) *CYB5*, *ACC1*$^{S659A, S1157A}$ (*S. cerevisiae*) *	CRISPR/Cas9-mediated gene integration (single copy)	Glucose	263.4
306	2020	*4CL1* (*P. appendiculatum*) *STS* (*P. cuspidatum*) + 5 other *STS*	Episomal plasmid	*p*-coumaric acid	39
302	2020	*STS* (*V. vinifera*) *TAL* (*T. cutaneum*) - in *E. coli*	Bacterial expression vectors	Glucose	36
		4CL (*A. thaliana*) - in *S. cerevisiae* *; Co-culture	Genomic integration (single copy)	*p*-coumaric acid (secreted from *E. coli*)	

* multiple modifications, refer to the original research article.

1.4.4.3.2. Other microorganisms

Among the non-*S. cerevisiae* microorganisms, *E. coli* is one of the most used hosts for the introduction of resveratrol biosynthetic pathways (Table **1.4**). In parallel with the aforesaid integration of *Nt4CL2* and *VvSTS* in *S. cerevisiae*, Beekwilder et al.[288] introduced the same genes in *E. coli* and achieved a production of 16 mg/L of resveratrol in the same rich medium (with 820.8 mg/L *p*-coumaric acid). In another study, by expression *At4CL1* and *AhSTS* in an *E. coli* strain, 104.5 mg/L of resveratrol was obtained on mineral

medium supplemented with 164 mg/L *p*-coumaric acid.[307] Lim et al. reported the highest titre in literature for *E. coli* fermentation, achieving 2.3 g/L of resveratrol from 2.46 g/L *p*-coumaric acid supplemented with 0.05 mM cerulenin, to reduce carbon loss from the malonyl-CoA to fatty acid production pathway. This was accomplished by the expression of *At4CL* and *VvSTS* after screening seven resveratrol synthases from different microorganisms.[308] Other studies reported resveratrol production in *E. coli* from tyrosine by using either *PAL*[309] or *TAL*[310], achieving similar titres of 37 and 35 mg/L of resveratrol from 540 and 544 mg/L tyrosine, respectively, while coupling them with different additional modifications. Resveratrol production in *E. coli* from glucose was firstly reported through site-specific integration of *RgTAL*, *Pc4CL* and *VvSTS* into the loci of the genes *tyrR*, a transcriptional regulatory gene, and *trpED*, encoding anthranilate synthetase. The inactivation of these genes enhances the expression levels of several genes in the shikimate pathway and abolishes the competing production of L-tryptophan, respectively. The authors reported a resveratrol concentration of 4.612 mg/L using the final recombinant strain.[311] Wu et al.[312] extensively engineered an *E. coli* strain by constructing a *de novo* resveratrol synthetic pathway from tyrosine (*TAL* from *Trichosporon cutaneum*, *Pc4CL* and *VvSTS*) and engineering the availability of malonyl-CoA using CRISPR interference (CRISPRi) and rational of engineering of tyrosine ammonia lyase through the reduction of mRNA secondary structure in its 5′ region. Altogether, the final strain was able to produce 304.5 mg/L of resveratrol from 5 g/L glucose, though the strain was unable to consume all the glucose in the medium. Modular engineering strategies have also been reported using *E. coli-E. coli* consortia. Resveratrol production from glycerol[313] and glucose[314] was reported, with titres of 22.6 and 55.7 mg/L of resveratrol, respectively. Both studies used similar strategies relying on the division of the biosynthetic pathway in two strains, where the first produced *p*-coumaric acid and the second converted the excreted *p*-coumaric acid into resveratrol. More recently, another *E.coli-E. coli* co-culture was used to produce resveratrol from glucose and arabinose, using a combination of CRISPR/Cas9 and CRISPRi technologies, where the first module was engineered to have deficient arabinose utilisation, producing *p*-coumaric acid from glucose, and the second consumed arabinose, glucose and the excreted *p*-coumaric acid, leading to a resveratrol titre of 204.8 mg/L.[315]

Recently, *Yarrowia lipolytica* has been gaining attention for resveratrol production processes, and after Gu et al.[316] reported 12.67 mg/L of resveratrol from glucose by expression and deletion of more than a dozen genes, Sáez-Sáez et al.[166] reported the production of 12.4 g/L of resveratrol in fed-batch fermentation from glucose, to date, the highest resveratrol titre in literature with any host (Table **1.4**). The authors expressed 5 copies of the resveratrol synthetic pathway comprising *TAL* from *Flavobacterium johnsoniae*, alongside *YlARO4*^{K221L}/*YlARO7*^{G139S}, mutated feedback-insensitive versions of DAHP synthase

and chorismite mutase, respectively. *S. stipitis*, due to its ability to naturally metabolise several carbon sources has been also used as a host for *de novo* resveratrol from different sugars like the monosaccharides glucose (237.6 mg/L), fructose (204.6 mg/L), galactose (170.6 mg/L), xylose (248.6 mg/L) or maltose (206.9 mg/L) and the disaccharides cellobiose (529.8 mg/L) and sucrose (668.6 mg/L), with 50 g/L of initial sugar concentration in all of them.[317] The authors introduced essential genes for resveratrol biosynthesis, namely *TAL* from *Herpetosiphon aurantiacus*, *At4CL2* and *VvVST1*, alongside a feedback-insensitive allele of chorismate mutase (*SsARO7*^{G1395}). Other hosts have been used for the biosynthesis of resveratrol either from precursors like *p*-coumaric acid/tyrosine or from carbon sources like glucose or glycerol, namely *Streptomyces venezuelae,*[318] *Lactococcus lactis,*[319]*, Ogataea polymorpha*[320] or *Corynebacterium glutamicum*.[321,322]

Table 1.4. Successful cases of microbial production of resveratrol using different non-*S. cerevisiae* hosts.

Microbial host	Relevant genotype	Substrate/ precursor	Titre (mg/L)	Year	Ref.
Bacteria					
E. coli	*4CL* (*N. tabacum*) *STS* (*V. vinifera*)	*p*-coumaric acid	16	2006	288
E. coli	*4CL* (*A. thaliana*) *STS* (*A. hypogaea*)	*p*-coumaric acid	105	2006	307
E. coli	*PAL* (*R. rubra*) *4CL* (*L. erythrorhizon*) *STS* (*A. hypogaea*) *ACC* (*C. glutamicum*)	Tyrosine	37	2007	309
E. coli	*4CL* (*L. erythrorhizon*) *STS* (*A. hypogaea*) *ACC* (*C. glutamicum*)	*p*-coumaric acid	171	2007	323
E. coli	*TAL* (*S. espanaensis*) *4CL* (*S. coelicolor*) *STS* (*A. hypogaea*)	*p*-coumaric acid	1.4	2011	324
E. coli	*4CL* (*A. thaliana*) *STS* (*V. vinifera*)	*p*-coumaric acid	2300	2011	308
E. coli	*TAL* (*R. glutinis*) *4CL* (*P. crispum*) *STS* (*V. vinifera*) *matB, matC* (*R. trifolii*)	Tyrosine	35.02	2013	310
E. coli	4CL (*A. thaliana*) STS (*V. vinifera*) *	*p*-coumaric acid	1600	2013	325
E. coli	*TAL* (*S. espanaensis*) *4CL* (*S. coelicolor*) *STS* (*A. hypogaea*)	Glucose	5.2	2014	326

Table 1.4. (continued)

Microbial host	Relevant genotype	Substrate/ precursor	Titre (mg/L)	Year	Ref.
E. coli	*4CL* (*P. crispum*) *STS* (*V. vinifera*) *fabD* downregulation	*p*-coumaric acid	268.2	2015	327
E. coli	*4CL::STS* (*A. thaliana, A. hypogaea*)	*p*-coumaric acid	80.5	2015	328
E. coli	*TAL* (*S. espanaensis*) *4CL* (*A. thaliana*) *STS* (*A. hypogaea*)	Tyrosine	114.4	2015	329
E. coli	*4CL* (*A. thaliana*) *STS* (*A. hypogaea*)	*p*-coumaric acid	160	2015	330
E. coli	*TAL* (*R. glutinis*) *4CL* (*P. crispum*) *STS* (*V. vinifera*) *ΔtyrR* and *ΔtrpED*	Glucose	4.612	2016	311
E. coli	*TAL* (*T. cutaneum*) *4CL* (*P. crispum*) *STS* (*V. vinifera*) *matB, matC* (*R. trifolii*) *	Glucose	304.5	2017	312
E. coli	*TAL* (*Saccharothrix espanaensis*) *4CL* (*Ocimum sanctum*) *STS* (*V. vinifera*) *	Glycerol	80.4	2021	331
E. coli-E.coli (co-culture)	*TAL* (R. glutinis) *tktA*^{fbr}, *aroG*^{fbr} (*E. coli*) *ΔpheA*	Glycerol	22.6	2016	313
	4CL (*Streptomyces coelicolor*) *STS* (*V. vinifera*)	*p*-coumaric (excreted)			
E. coli-E. coli (co-culture)	*TAL* (R. glutinis) *tktA*^{fbr}, *aroG*^{fbr} (*E. coli*) *ΔpheA, Δpgi*	Glucose	55.7	2020	314
	4CL (*Petroselinum crispum*) *STS* (*A. hypogaea*) *ACC* (*C. glutamicum*) *Δzwf*	*p*-coumaric (excreted)			
E. coli-E. coli (co-culture)	*TAL* (*Phanerochaete chrysosporium*) *	Glucose	204.80	2022	315
	4CL (*A. thaliana*) *STS* (*V. vinifera*) *	Arabinose, *p*-coumaric acid (excreted)			

Table 1.4. (continued)

Microbial host	Relevant genotype	Substrate/ precursor	Titre (mg/L)	Year	Ref.
C. glutamicum	*STS* (*A. hypogaea*) *4CL* (*P. crispum*) *	*p*-coumaric acid	158	2016	321
C. glutamicum	*TAL* (*F. johnsoniae*) *4CL* (*P. crispum*) *STS* (*A. hypogaea*) *aroH* (*E. coli*) *	Glucose	12	2018	322
L. lactis	*TAL, 4CL, STS, ACC* (multiple sources)	Glucose	1.27	2016	319
Yeast					
Y. lipolytica	*TAL* (*R. toruloides*) *4CL* (*P. crispum*) *VST* (*V. vinifera*) *ARO4*^{fbr} (*S. cerevisiae*) *	Glucose	12.67	2020	316
Y. lipolytica	*4CL* (*N. tabacum*) *STS* (*A. hypogaea*) *PEX10, ACC1 (Y. lipolytica)*	*p*-coumaric acid	48.7	2020	332
Y. lipolytica	*TAL* (*F. johnsoniae*) *PAL* (*V. vinifera*) *C4H*, *4CL1* (*A. thaliana*) *VST* (*V. vinifera*)	Glycerol	430	2020	333
Y. lipolytica	*TAL* (*F. johnsoniae*) *4CL* (*A. thaliana*) *VST* (*V. vinifera*) *ARO4*^{fbr}, *ARO7*^{fbr} (*Y. lipolytica*)	Glucose	12355	2020	166
S. venezuelae	*STS* (*A. hypogaea*) *4CL* (*S. coelicolor*) *Δpks*	*p*-coumaric acid	0.4	2009	318
O. polymorpha	*TAL (H. aurantiacus)* *4CL (A. thaliana)* *STS (V. vinifera)*	Tyrosine	97.23	2018	320
S. stipitis	*TAL* (*H. aurantiacus*) *4CL2* (*A. thaliana*) *VST* (*V. vinifera*) *ARO7*^{G139S} (*S. stipitis*)	Glucose Fructose Galactose Xylose Maltose Cellobiose Sucrose	237.6 204.6 170.6 248.6 206.9 529.8 668.6	2021	317

* multiple modifications, refer to the original research article.

1.5. Aim of the thesis

Despite the urgent need to produce high-value chemicals from biomass, achievements in this regard have been rather scarce. All the studies reported in the literature so far establish resveratrol bioprocesses on synthetic media, with the vast majority recurring to laboratory *S. cerevisiae* or other microorganisms that do not cope well with the challenges associated with lignocellulosic fermentation. The use of renewable carbon sources can contribute to a more sustainable biorefinery, increasing its economic viability by reducing operation costs, and promoting the concept of circular bioeconomy by reintroducing agro-industrial wastes back into the supply chain.

The main purpose of this thesis is to develop industrial yeast-based processes for *de novo* resveratrol production from lignocellulosic biomass and other agro-industrial wastes, for the development of an integrated and intensified process. Figure **1.9** shows a schematic of the overall integration of residues used in this study and its main steps. Resveratrol was previously reported to be produced from glucose and ethanol, which allows for channelling of the previously accumulated knowledge of lignocellulose-to-ethanol processes.[19,334] Nevertheless, no efforts have been made towards the utilisation of non-naturally metabolised carbon sources by *S. cerevisiae* for resveratrol biosynthesis. Here, we employed CRISPR/Cas9-based metabolic engineering strategies to generate yeast strains capable of utilising carbon sources lactose and xylose, which wild-type strains cannot achieve. Therefore, the main goals of this thesis are:

1. Identify robust industrial yeast candidates for their aptitude to produce resveratrol in process-like conditions such as high temperature;
2. Integration of the engineered yeast in the biomass-to-resveratrol process by utilising different LCM for resveratrol production, evaluating alternative configurations for more efficient processes;
3. Metabolic engineering of yeast strains for the consumption of non-naturally metabolised carbon sources such as lactose and xylose for the valorisation of other agro-industrial wastes such as cheese whey powder and winery by-products;
4. Evaluation of fermentation conditions for increased titres and yields through optimisation of the resveratrol bioprocess, through the combination of different conditions and carbon sources and assessment of alternative fermentation configurations.

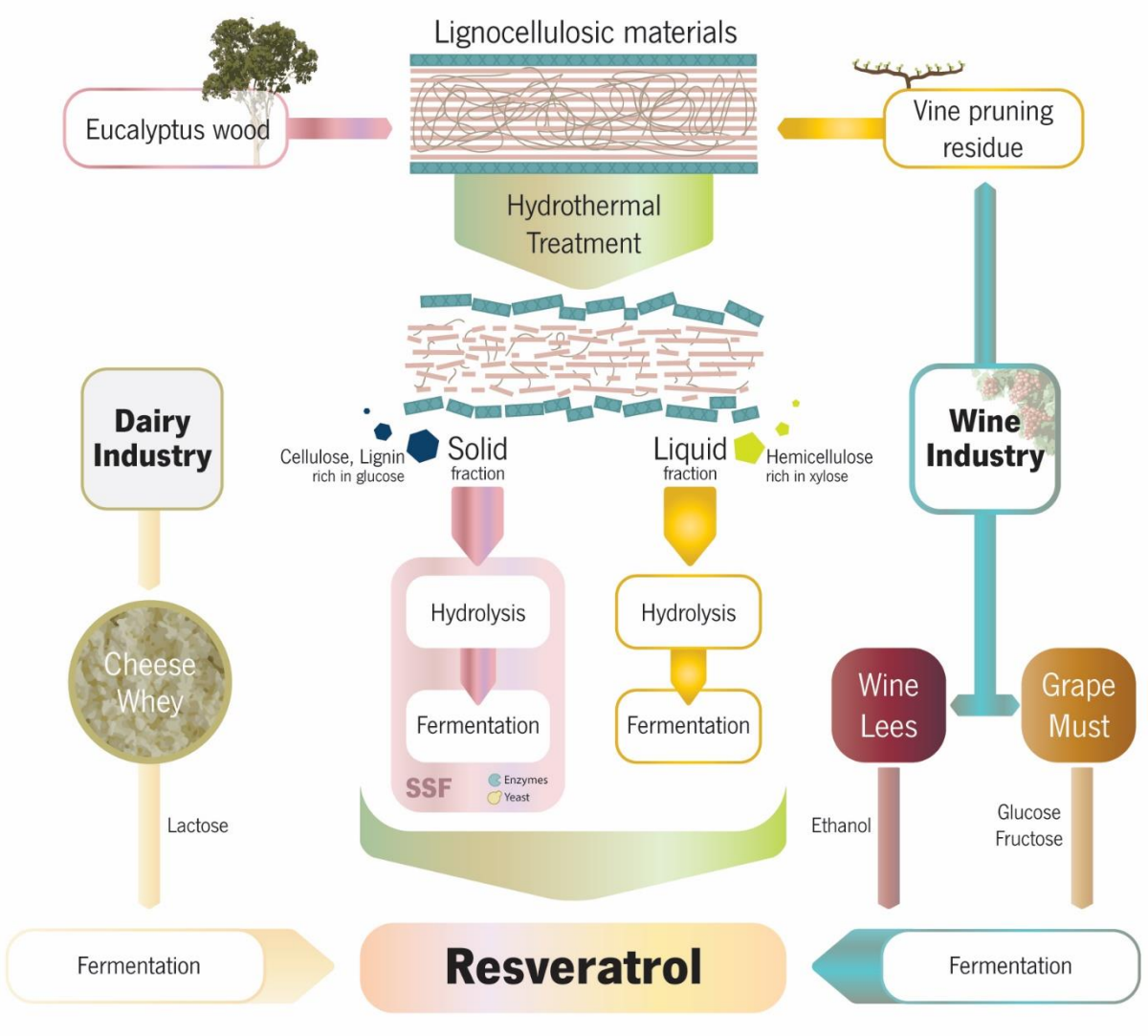

Figure 1.9. Schematic overview of the main objectives of the thesis.

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Chapter II.

Resveratrol production from hydrothermally pretreated Eucalyptus wood using recombinant industrial *Saccharomyces cerevisiae* strains

This chapter is based on the following original research article:

Costa, CE; Møller-Hansen, I; Romaní, A; Teixeira, JA; Borodina, I; Domingues, L. **2021**. Resveratrol production from hydrothermally pretreated Eucalyptus wood using recombinant industrial *Saccharomyces cerevisiae* strains. *ACS Synth. Biol. 10* (8). 1895–1903. DOI: 10.1021/acssynbio.1c00120.

Abstract

Resveratrol is a phenolic compound with strong antioxidant activity, being promising for several applications in health, food and cosmetics. It is generally extracted from plants or chemically synthesised, both complex and not sustainable processes, but microbial biosynthesis of resveratrol can side these drawbacks. In this work, resveratrol production by microbial biosynthesis from lignocellulosic materials was assessed. Three robust industrial *Saccharomyces cerevisiae* strains, known for their thermotolerance and/or resistance to inhibitory compounds, were identified as suitable hosts for *de novo* resveratrol production from glucose and ethanol. Through the CRISPR/Cas9 system, all industrial strains, and a laboratory one, were successfully engineered with the resveratrol biosynthetic pathway via phenylalanine intermediate. All strains were further screened at 30 °C and 39 °C to evaluate thermotolerance, which is a key feature for Simultaneous Saccharification and Fermentation processes. Ethanol Red RBP showed the best performance at 39 °C, with more than 2.6-fold of resveratrol production in comparison with the other strains. This strain was then used to assess resveratrol production from glucose and ethanol. A maximum resveratrol titre of 187.07 ± 19.88 mg/L was attained from a medium with 2% glucose and 5% ethanol (w/v). Lastly, Ethanol Red RBP produced 151.65 ± 3.84 mg/L of resveratrol from 2.95% of cellulose from hydrothermally pretreated *Eucalyptus globulus* wood, at 39 °C, in a Simultaneous Saccharification and Fermentation process. To the best of our knowledge, this is the first report on lignocellulosic resveratrol production, establishing grounds for the implementation of an integrated lignocellulose-to-resveratrol process in an industrial context.

Keywords: resveratrol, *Saccharomyces cerevisiae*, CRISPR/Cas9, eucalyptus wood

2.1. Introduction

Resveratrol is a phenolic compound with high commercial value, having strong antioxidant activity, with known anti-inflammatory and anti-ageing properties, among others, arising as a promising compound for applications in health, diet and cosmetics.[1] It is naturally produced in plants as a response to stress conditions,[2] and it is generally obtained from Japanese knotweed by chemical extraction processes.[3] Moreover, resveratrol can also be chemically synthesised, which has been widely reported for decades, with several methodologies available, such as Heck-, Perkin- or Wittig-reaction.[4–6] Though relatively high yields can be obtained, these processes are complex, involving multiple reactions and harsh conditions like very high temperatures, and extremely polluting.[7] Biotechnological production of resveratrol is an attractive alternative as it poses several advantages by being environmentally friendly, having low cost and enabling high product purity.[8] It has been mainly produced at the cost of expensive substrates such as *p*-coumaric acid,[9–11] but its *de novo* production has been reported and commercially explored in recent years using different hosts like *Escherichia coli;*[12] *Yarrowia lipolytica* [13] or *Saccharomyces cerevisiae.*[8,14] Resveratrol biosynthesis by these recombinant microorganisms can be attained through tyrosine or phenylalanine via the shikimate pathway. From here, the introduction of heterologous genes enables the conversion of these amino acids into phenylpropanoic acids, ultimately leading to resveratrol production from carbon sources like glucose and ethanol.[15]

In the quest for a biobased economy, there is an urgent need to show the feasibility of using cheap carbon sources like lignocellulosic materials to produce high-value compounds other than bioethanol.[16] Lignocellulosic biomass is the most abundant and available renewable resource, composed of 40-50% of cellulose, which is the most abundant polymer on Earth.[17] Cellulose is made of thousands of D-glucose molecules linked in a linear chain by β(1,4)-glycosidic bonds.[17] However, pretreatment is mandatory to break down the recalcitrant lignocellulosic structure, improving the enzymatic saccharification of cellulose to glucose production. In this sense, hydrothermal treatment (also known as liquid hot water or autohydrolysis), which uses water as a reaction medium, allows the solubilisation of hemicellulose as xylooligosaccharides and enables the recovery of lignin and cellulose in the solid phase.[18] Some advantages of this treatment include low degradation of inhibitory compounds, reduced corrosion, low energy requirements and no need for neutralisation steps.[19] Therefore, cellulosic fraction after hydrothermal treatment can be used for glucose and ethanol production by saccharification of cellulose into glucose and subsequent fermentation of glucose into ethanol.[20] Simultaneous Saccharification and Fermentation (SSF) processes are widely used for ethanol production, having several advantages for process development in comparison with separate hydrolysis and fermentation (SHF) such as avoidance

of end-product inhibition or reducing the required number of vessels.[21] Nevertheless, the main drawback of the SSF process is the selection of temperature to carry out both processes (saccharification and fermentation), since a temperature of 45-50 ºC is optimal for cellulase activity and 30 ºC for yeast growth.[22,23]

In this context, the advances in ethanol production by SSF processes can additionally be an attractive strategy for resveratrol biosynthesis, as *S. cerevisiae* can convert ethanol to acetaldehyde, which can then originate malonyl-CoA, a direct precursor of resveratrol.[8,14] An optimal SSF process is deeply connected to the efficient saccharification of cellulose into glucose. Given this, one of the most desirable properties of a fermentative microorganism for this process is thermotolerance, since it allows effective fermentation at near-optimal saccharification temperatures, increasing the overall efficiency of the process.[22]

The yeast *S. cerevisiae* is a microorganism widely used in several microbiological processes in several industries such as food, biofuel, nutraceutical and pharmaceutical.[16] Industrial *S. cerevisiae* isolates have received special attention due to their higher robustness, fermentation capacity and resistance to stress factors when compared with laboratory strains.[24] Appropriate selection of yeast chassis plays a major role in the development of an integrated sustainable lignocellulosic-based process,[18] where multiple stress factors and challenging conditions (e.g. high temperature, pentose sugar utilisation, presence of inhibitory compounds) are present simultaneously.[25,26] Here, three industrial robust *S. cerevisiae* strains, known for their thermotolerance and/or resistance to inhibitory compounds,[18,27,28] together with a control laboratory strain, were engineered, and their aptitude for *de novo* resveratrol production from glucose or ethanol was evaluated. Moreover, for the first time, resveratrol production was attained from lignocellulose, in an SSF process.

2.2. Materials and Methods

2.2.1. Strains and plasmids

Cloning work was performed using *E. coli* strains DH5α competent cells (prepared in-house). *E. coli* transformants were selected and maintained on Lysogeny Broth (LB) plates with 100 mg/L of ampicillin. Plasmid construction was made by USER cloning, as described in Jensen et al.[29] All plasmids used are listed in Appendix: Table A2.1. All primers used in this work and the biobrick generated are listed in Table A2.2 and Table A2.3, respectively. Biobrick assembly was made in an EasyClone-MarkerFree vector[30] to create the integrative plasmid for the desired gene overexpression. Correct cloning was confirmed by sequencing. Yeast transformations were accomplished following the lithium acetate protocol and the successful integration of genes was confirmed by yeast colony PCR.[31] All strains were initially transformed with a Cas9-expressing plasmid and selected on G418 plates. The resulting strains expressing the Cas9 protein were subsequently transformed with the desired DNA fragment together with the corresponding guide RNA (gRNA) plasmid to target the selected insertion site.

Three industrial diploid *S. cerevisiae* strains were used as chassis strains in this work: PE-2 and CAT-1, isolated from Brazilian first-generation bioethanol plants,[32] and Ethanol Red®, a commercial strain developed by Fermentis, S.I. Lesaffre for the ethanol industry. Additionally, the laboratory haploid strain CEN.PK113-7D was used as control. These strains were selected stemming from the knowledge acquired during previous research work in our research group (within the ERA-net project Yeastemptation), where the temperature range was defined up to 39 °C, the threshold for cell growth of the control strain.[28] Therefore, 39 °C was the temperature chosen for this set of experiments at "high temperature". By the introduction of the resveratrol biosynthetic pathway (RBP), the four recombinant strains used in this study were generated: CEN.PK113-7D RBP, PE-2 RBP, CAT-1 RBP and Ethanol Red RBP.

2.2.2. Media and cultivations

Yeast strains carrying the Cas9 plasmid were kept in YPD plates (1% yeast extract, 2% peptone, 2% dextrose, 1.5% agar) with 200 mg/L of G418. YPD plates with 200 mg/L of G418 and 100 mg/L of nourseothricin (clonNAT) were used for CRISPR/Cas9 transformations. For all assays, pre-cultures of yeast cells for inoculation were grown overnight in 250 mL baffled shake flasks with a working volume of 50 mL of YPD20 (1% yeast extract, 2% peptone, 2% dextrose), with orbital shaking (300 rpm) at 30 °C. Percentages are weight per volume (w/v).

2.2.3. Resveratrol production in synthetic media and SSF of pretreated *Eucalyptus globulus* wood

All assays were carried out in an orbital shaker (300 rpm) at 30 °C or 39 °C, with biological duplicates or triplicates. Fermentations were monitored by sample collection. For experiments carried out in shake flasks, at each timepoint, 500 µL of the sample was centrifuged at 13,000 rpm for 5 min and the supernatant was stored for further analysis of sugars, glycerol, acetic acid and ethanol. Another 500 µL of the sample was mixed with an equal volume of ethanol (>99% purity), vortexed and centrifuged at 13,000 rpm for 5 min, and the supernatant was analysed for resveratrol and *p*-coumaric acid quantification.

2.2.3.1. Synthetic media

For evaluation of resveratrol production from glucose, a 96-deep-well microtitre plate with an air-penetrable lid (EnzyScreen, NL), in YPD20 media, with a working volume of 500 µL, were used. The assay was carried out for 72 h and the final samples were diluted in an equal volume of ethanol (>99% purity) and the supernatants were analysed for resveratrol concentration. For thermotolerance evaluation assays, baffled shake flasks of 250 mL with 50 mL of working volume were used. For assessment of ethanol as substrate, shake flasks of 100 mL with 20 mL of working volume were used. YPD20 media was supplemented with different percentages of ethanol (>99% purity) in weight per volume (w/v). For all the above, OD_{600} of the pre-cultures was adjusted to 1 and all media were inoculated (10% v/v) to obtain an initial OD_{600} of 0.1.

2.2.3.2. Simultaneous saccharification and fermentation of pretreated EGW

For SSF assays of hydrothermally pretreated *Eucalyptus globulus* wood (EGW), two solid EGW loadings were used, 5% and 10%, with the addition of cellulase (Cellic CTec2) at 20 FPU of cellulase/g of autohydrolysed EGW. These operational conditions were selected based on previously published works by the authors.[33,34] Cellic Ctec2 was kindly provided by Novozyme (Bagsvaerd, Denmark) and its activity (122 FPU/mL) was measured by the method described by Ghose et al.[35] Cells from pre-culture were recovered by centrifugation (5 min, 4000g). Pellets were resuspended in 0.9% (w/v) sodium chloride solution for an inoculum concentration of 8 mg of fresh yeast/mL in the SSF assay.[18]

2.2.4. Pretreated Eucalyptus globulus wood

The raw material used in this work was *Eucalyptus globulus* wood, provided by a local pulp mill (ENCE, Galicia, Spain). EGW was submitted to hydrothermal treatment. For that, EGW was mixed with water at a liquid-solid ratio of 8 g/g in a pressurised Parr reactor and heated at the desired temperature of 210 °C (or severity factor S_0 of 4.08). Conditions of pretreatment were selected based on the literature[40]. The chemical composition of raw material and pretreated *Eucalyptus globulus* wood using NREL procedures were previously reported by Costa et al.[18] Hydrothermally pretreated EGW was composed (measured as g/100 g of pretreated EGW in oven-dry basis ± standard deviation is as follows: 59% ± 0.23% of glucan, 2.1% ± 0.11% of xylan and 34% ± 0.40% of Klason lignin. The solid fraction of this pretreated biomass was used as the substrate for resveratrol production by SSF.

2.2.5. Analytical methods

Samples collected from fermentation runs were analysed for glucose, xylose, glycerol, acetic acid and ethanol concentrations by HPLC using a BioRad Aminex HPX-87H column, at 60 °C, with a mobile phase 5 mM H_2SO_4 and flow rate of 0.6 mL/min. The peaks were detected using a Knauer-IR intelligent refractive index detector. Resveratrol and *p*-coumaric acid were quantified by UHPLC equipped with a Discovery® HS F5 150 mm × 2.1 mm column (particle size 3 mm). The eluent flow rate was 0.7 mL/min. A linear gradient from 5% to 60% of acetonitrile over 10 mM ammonium formate (pH 3.0, adjusted by formic acid) from 0.5–9.5 min was used. Resveratrol was detected by absorbance at 304 nm with a retention time of 7.4 min and *p*-coumaric acid at 277 nm, with a retention time of 5.6 min. The OD_{600} was measured on a Genesys 20 Spectrophotometer (Thermo Scientific). Biomass dry weight (BDW) quantification was made by collecting 1 mL of fermentation broth to previously dried and weighted tubes, washed out twice in ethanol and deionised water, sequentially, and incubated at 105 °C for 24 h and weighed again. Biomass quantification in the fermentation of pretreated EGW was not attained due to the impossibility of separation between yeast cells and lignocellulosic biomass in suspension.

2.2.6. Determination of fermentation parameters

Resveratrol yield on biomass dry weight ($Y_{R/BDW}$) was calculated as the ratio between resveratrol and biomass concentrations at the end of fermentation. Resveratrol yield on glucose ($Y_{R/G}$) was calculated by the ratio between the resveratrol concentration and the amount of glucose consumed at the end of cultivation. The productivity of resveratrol at 24 h of fermentation (Q_{R24}) was calculated as the ratio between

resveratrol produced at 24 h of fermentation and that time (24 h). Potential glucose (G_{POT}) obtained from the cellulose of pretreated biomass was calculated as follows:

$$G_{POT} = \frac{Gn}{100} \cdot \frac{180}{162} \cdot \frac{\rho}{\frac{1}{\%\ solid} - \frac{KL}{100}}$$

where Gn is the glucan content of pretreated EGW, 180/162 is the stoichiometric factor for glucan hydration upon hydrolysis, ρ is the density of the reaction medium, and % solid is the concentration of pretreated EGW in the SSF process and KL is the content in lignin of pretreated EGW.

2.2.7. Statistical analysis

GraphPad Prism for Windows version 8.02 was used to carry out the statistical analyses. Differences between the strain profiles in terms of sugar and ethanol concentrations, biomass formation and resveratrol production were tested by repeated measures two-way ANOVA, followed by Tukey post hoc test. Statistical significance was established at *p*-value < 0.05 for the comparisons and marked by "ns" (non-significant) - $p > 0.05$; * - $p < 0.05$; ** - $p < 0.01$; *** - $p < 0.001$; **** - $p < 0.0001$

2.3. Results and Discussion

2.3.1. Engineering industrial *S. cerevisiae* strains for *de novo* resveratrol production from glucose

The resveratrol biosynthetic pathway (RBP) used in this work leads to resveratrol formation from glucose or ethanol through phenylalanine and encompasses four genes: phenylalanine ammonia lyase (*AtPAL2*), cinnamic acid hydroxylase (*AtC4H*) and *p*-coumaryl-CoA ligase (*At4CL2*) from *Arabidopsis thaliana* and resveratrol synthase (*VvVST1*) from *Vitis vinifera* (Figure **2.1a**).

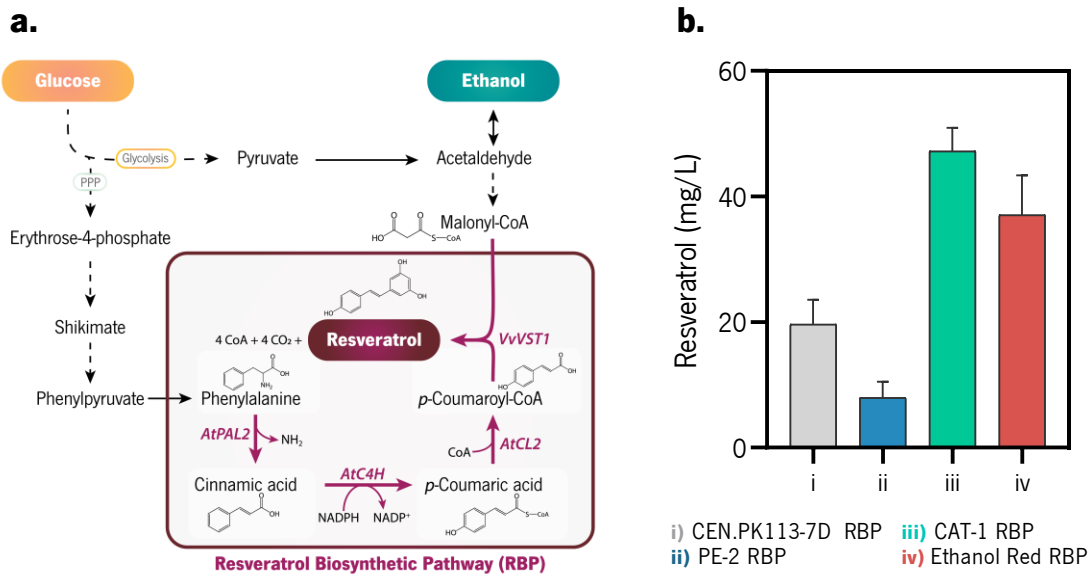

Figure 2.1. a) Overview of the *de novo* resveratrol biosynthesis in four recombinant *S. cerevisiae* strains. Single arrows represent single reaction steps and dashed arrows represent multiple reaction steps. Bold purple arrows indicate the reactions catalysed by the heterologous genes expressed, constituting the Resveratrol Biosynthetic Pathway (RBP): *AtPAL2*, phenylalanine ammonia lyase from *A. thaliana*; *AtC4H*, cinnamic acid hydroxylase from *A. thaliana*; *At4CL2*, *p*-coumaroyl-CoA ligase from *A. thaliana*; *VvVST1*, resveratrol synthase from *V. vinifera*. PPP; Pentose Phosphate Pathway. **b)** Resveratrol production by the four engineered yeast strains expressing the RBP genes after 72 hours of cultivation in 96 microtitre deep-well plates in YPD medium (2% glucose). Each column represents the average values ± standard deviation of biological triplicates. Statistical analysis: i vs. ii*; i vs. iii***; i vs. iv**; ii vs. iii****; ii vs. iv***; iii vs. iv – ns.

Its introduction in the selected *S. cerevisiae* strains was made under the action of two strong constitutive promoters, *pTEF1* and *pPGK1*, as reported by Li et al.[14] The RBP was integrated into all 4 strains in a single transformation into the same selected insertion site (Appendix: Table A2.1). This was

successfully accomplished due to 100% homology of the guide RNA and >99% homology of the flanking recombinant regions with this location in chromosome X on all strains. Nevertheless, while CEN.PK113-7D, CAT-1 and Ethanol Red showed a transformation efficiency of 100%, this value was lower than 10% for PE-2. The mechanisms behind this are not clear but may highlight the heterogeneity associated with the different backgrounds when considering industrial yeast strains. All recombinant strains studied showed *de novo* resveratrol production from glucose (Figure **2.1b**). CAT-1 RBP and Ethanol Red RBP, the top-producing strains, produced 47.24 ± 3.68 mg/L and 37.05 ± 6.33 mg/L of resveratrol, respectively, though no statically significant differences were observed between them. The lower resveratrol production was observed in PE-2 RBP, attaining only 7.96 ± 2.51 mg/L of resveratrol, while the laboratory strain CEN.PK113-7D RBP produced 19.64 ± 3.89 mg/L of resveratrol. The overall higher titre of resveratrol observed in industrial strains might also be related to its diploidicity, as the RBP was introduced in two copies in these strains, against only one in the laboratory haploid strain (Appendix: Figure A2.1). As shown previously by Li et al.,[8] integration of the resveratrol biosynthetic pathway in multiple copies is associated with an improvement in resveratrol production.

2.3.2. Evaluation of thermotolerance for process-like conditions for resveratrol production

Taking into account the results displayed in Figure **2.1**, all recombinant strains were used to evaluate their behaviour at different temperatures of fermentation. Therefore, the four previously engineered strains were cultivated at 30 °C and 39 °C (Figure **2.2**). In comparison with the results from Figure **2.1b**, Figure **2.2a** shows that the shift from 96-deep-well microtitre plates to shake flasks led to more than 2-fold increase in resveratrol production at 30 °C in all strains tested (more than 4-fold in PE-2 RBP). This may be explained by better oxygenation conditions, which are long-known to have a positive influence on biomass formation,[36] as resveratrol accumulation is deeply related to cell growth, requiring large amounts of ATP (approx. 13 mole of ATP per mole of resveratrol).[37] Still, the same ranking for resveratrol production was observed at 30 °C among the strains (Figure **2.2a**). CAT-1 RBP and Ethanol Red RBP were the top-producing strains, with a titre of 102.59 ± 5.77 mg/L and 86.18 ± 5.73 mg/L of resveratrol, respectively, while CEN.PK113-7D RBP produced 49.83 ± 0.47 mg/L of resveratrol, followed by PE-2 RBP with 33.13 ± 1.73 mg/L of resveratrol, with statistically significant differences ($p < 0.05$) between all of them. No residual ethanol was observed at the end of fermentation (Figure **2.2b**). All glucose was depleted in the first 24 h for all strains (data not shown).

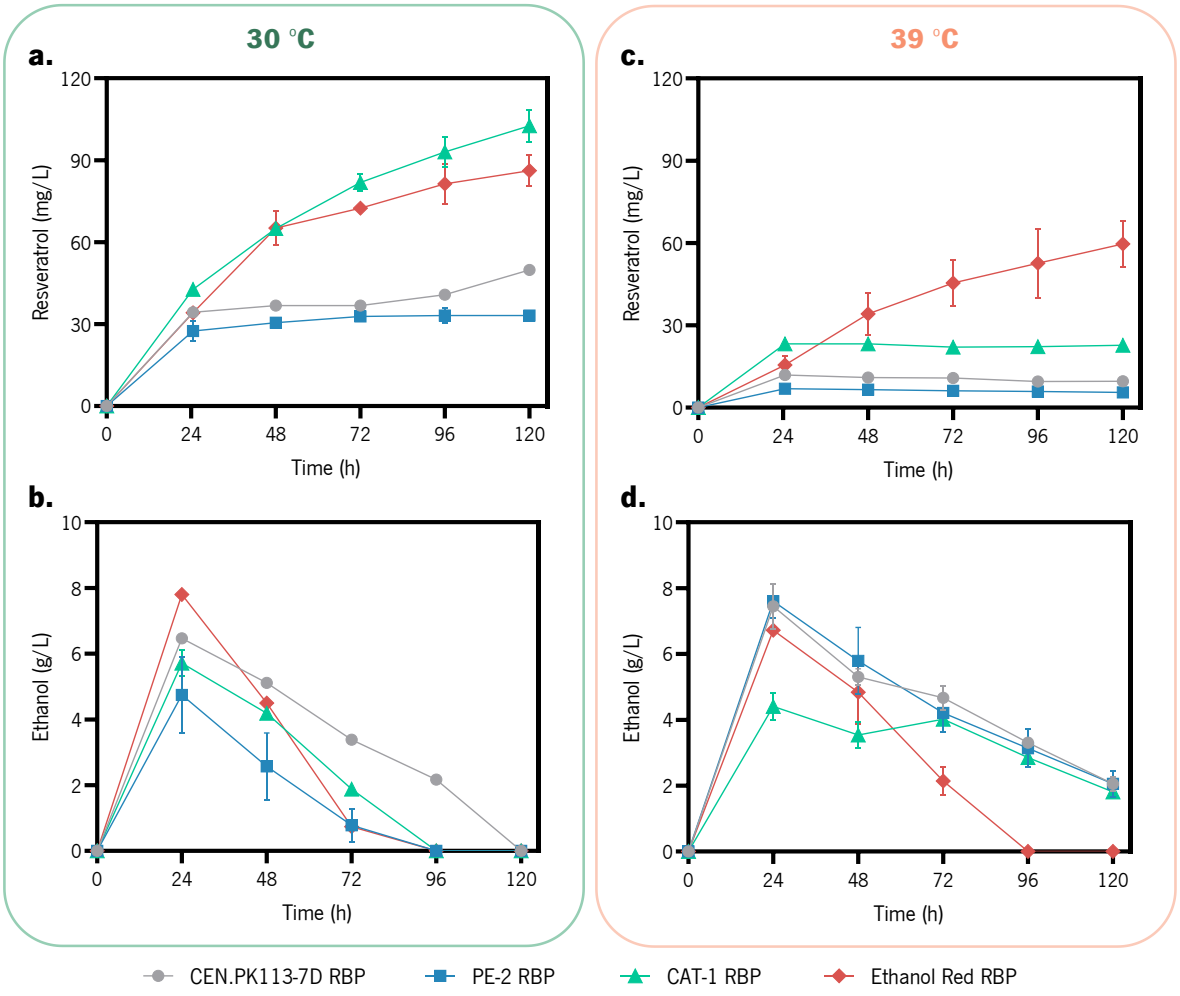

Figure 2.2. Time course of resveratrol (**a, c**) and ethanol (**b, d**) concentrations at 30 °C (**a, b**) and 39 °C (**c, d**) in YPD media (2% glucose) for CEN.PK113-7D RBP (grey circles), PE-2 RBP (blue squares), CAT-1 RPC (green triangles) and Ethanol Red RBP (red diamonds) fermentations. Each data point represents the average ± standard deviation of biological duplicates. Statistical analysis at t = 120h: a) all data points are statistically significant from each other (***); b) no statistically significant differences; c) all data points are statistically significant from each other (p < 0.05 or less), except between CEN.PK113-7D RBP and PE-2 RBP; d) statistically significant differences between Ethanol Red RBP and the other three strains (****), but no statistically significant differences between CEN.PK113-7D, PE-2 RBP and CAT-1 RBP.

On the other hand, the rise in temperature to 39 °C clearly revealed Ethanol Red RBP as the best strain for high-temperature fermentation, attaining 59.64 ± 8.48 mg/L of resveratrol (Figure **2.2c**), more than 2.6-fold in comparison with the other strains. This is in accordance with previous findings regarding the thermotolerance of this strain background.[28] CAT-1 RBP, which also derives from a thermotolerant strain,[18,32] produced 22.73 ± 0.20 mg/L of resveratrol. As shown in Table **2.1**, although the yield on

resveratrol per biomass dry weight showed no statistically significant differences between these two strains, Ethanol Red RBP yield on initial glucose fed was 2.6-fold higher than CAT-1 RBP. In the first 24 h of fermentation, resveratrol production by CAT-1 RBP was slightly higher than the other strains, exhibiting lower ethanol concentration in the fermentation media. However, this strain appears to be inhibited by fermentation conditions, ceasing resveratrol production after that timepoint. It is known that resveratrol production affects yeast physiology,[37] which is linked to high-temperature stress might be amplified since at 30 ºC this strain is capable to produce 4.5-fold more resveratrol. This highlights the need for a robust strain able to cope with simultaneous fermentation inhibitors. The other two strains did not reach 10 mg/L of resveratrol at 39 °C. At this temperature, even though ethanol concentration decreased after 24 h in all strains, only Ethanol Red RBP was able to produce resveratrol after this timepoint. It is possible that ethanol decrease in the other three strains may be accounted for cell growth and maintenance, as well as its partial evaporation. No *p*-coumaric acid formation was detected at any point, at both temperatures, for all strains, suggesting that all the *p*-coumaric acid produced is converted to resveratrol. Temperature increase led also to a reduction in biomass formation (Table **2.1**), where Ethanol Red RBP showed around 2.5-fold more biomass in dry weight than the other three strains. Previous findings also reported that Ethanol Red is more prone to biomass formation than CEN.PK113-7D, reaching higher yields of biomass per glucose (1.2- to 1.3-fold, both at 30 and 39 °C), showing also higher maximum specific growth rates, especially at 39 °C.[28] The same study also shows that, at 39 °C, the yield of biomass on ATP of Ethanol Red is 2-fold higher than CEN.PK113-7D,[28] which is crucial for resveratrol production. An increase in cultivation temperature is also known to correlate positively with maintenance energy requirements.[38] According to Lip et al., an increase in cultivation temperature from 30 °C to 39 °C would increase the maintenance coefficient by 2.2-fold, decreasing the yield on ATP.[28] The authors reported that, while CEN.PK113-7D decreased its biomass yield on ATP by more than 40%, this increase in cultivation temperature led to a reduction of only 13% in Ethanol Red, clearly showing its better aptitude for higher temperature cultivation. Additionally, Ethanol Red RBP was the only strain that did not accumulate residual ethanol at the end of fermentation at 39 °C (Figure **2.2d**). Ethanol Red response to this supra-optimal temperature was found to trigger mechanisms like repression of proteins involved in arginine biosynthesis (Arg1, Arg5, Arg6 and Arg8) and induction of a protein (Car2). The latter catalyses arginine degradation, guaranteeing glutamate conservation and proline production, which is thought to be involved in stress protection.[39] This, though, does not occur in CEN.PK113-7D.[39] This response is also observed in the yeast *Kluyveromyces marxianus*, known for its thermotolerance, when cultivated at 45 °C.[40] Interestingly, the strains that showed lower resveratrol titre at this temperature

exhibited the highest ethanol concentration at 24 h, when all glucose was consumed. Previous findings reported the positive effect of low resveratrol concentrations in suppressing ethanol stress in *S. cerevisiae* by affecting superoxide dismutase activity, lipid peroxidation and fatty acid profiles,[41] thus enabling higher ethanol production.

Table 2.1. Fermentation parameters of the four recombinant yeast strains in YPD media at 30 °C and 39 °C.

ID	Strain	T (°C)	G_{t0} (g/L)	E_{tf} (g/L)	R_{tf} (mg/L)	BDW (g/L)	$Y_{R/BDW}$ (mg/g)	$Y_{R/G}$ (mg/g)	Q_{R24} (mg/L·h)
	CEN.PK113-7D RBP								
1		30	19.17 ± 0.52	0.00 ± 0.00	49.83 ± 0.47	5.0 ± 0.6	10.03 ± 1.23	2.60 ± 0.05	1.40 ± 0.05
2		39	20.20 ± 0.23	2.06 ± 0.21	9.57 ± 1.27	1.2 ± 0.1	8.10 ± 2.01	0.47 ± 0.07	0.49 ± 0.05[1]
	PE-2 RBP								
3		30	19.17 ± 0.52	0.00 ± 0.00	33.13 ± 1.73	4.2 ± 0.6	7.93 ± 0.66	1.73 ± 0.04	1.12 ± 0.16
4		39	20.20 ± 0.23	2.06 ± 0.40	5.52 ± 0.71	1.1 ± 0.1	5.02 ± 0.00	0.27 ± 0.04	0.28 ± 0.03
	CAT-1 RBP								
5		30	19.17 ± 0.52	0.00 ± 0.00	102.59 ± 5.77	4.0 ± 0.2	26.05 ± 2.86	5.35 ± 0.16	1.74 ± 0.03
6		39	20.20 ± 0.23	1.81 ± 0.06	22.73 ± 0.20	1.4 ± 0.1	16.86 ± 1.03	1.13 ± 0.02	0.95 ± 0.01
	Ethanol Red RBP								
7		30	19.17 ± 0.52	0.00 ± 0.00	86.18 ± 5.73	3.8 ± 0.2	23.06 ± 2.83	4.49 ± 0.18	1.39 ± 0.10
8		39	20.20 ± 0.23	0.00 ± 0.00	59.64 ± 8.48	3.4 ± 0.4	17.92 ± 3.67	2.96 ± 0.45	0.63 ± 0.14

T (°C), Temperature; G_{t0}, initial glucose concentration; E_{tf}, final ethanol concentration; R_{tf}, final resveratrol concentration; BDW, Biomass Dry Weight; $Y_{R/BDW}$, Yield of resveratrol per biomass dry weight; $Y_{R/G}$; yield of resveratrol per initial glucose fed to the medium; Q_{R24}, productivity of resveratrol at 24 h of fermentation.

2.3.3. Evaluation of the effect of ethanol supplementation on resveratrol production

Considering the superior fermentation performance of Ethanol Red RBP at high temperature, this strain was selected for the subsequent assays. Ethanol, the natural product of alcoholic fermentation in *S. cerevisiae,* can act as a substrate for resveratrol *de novo* production (Figure **2.1a**). The ethanol phase of resveratrol production is known to lead to higher product yields than the glucose phase.[8,14] This can also be observed for Ethanol Red RBP in Figure **2.2** where after 24 h of fermentation, no glucose is present in the media, but resveratrol production increases more than 2-fold by consuming the ethanol in the media. Given this, tolerance to ethanol and aptitude to use it as a substrate of Ethanol Red RBP was evaluated. For that, a range of concentrations from 0% to 8% of ethanol was supplemented with YPD20 media (Figure **2.3**).

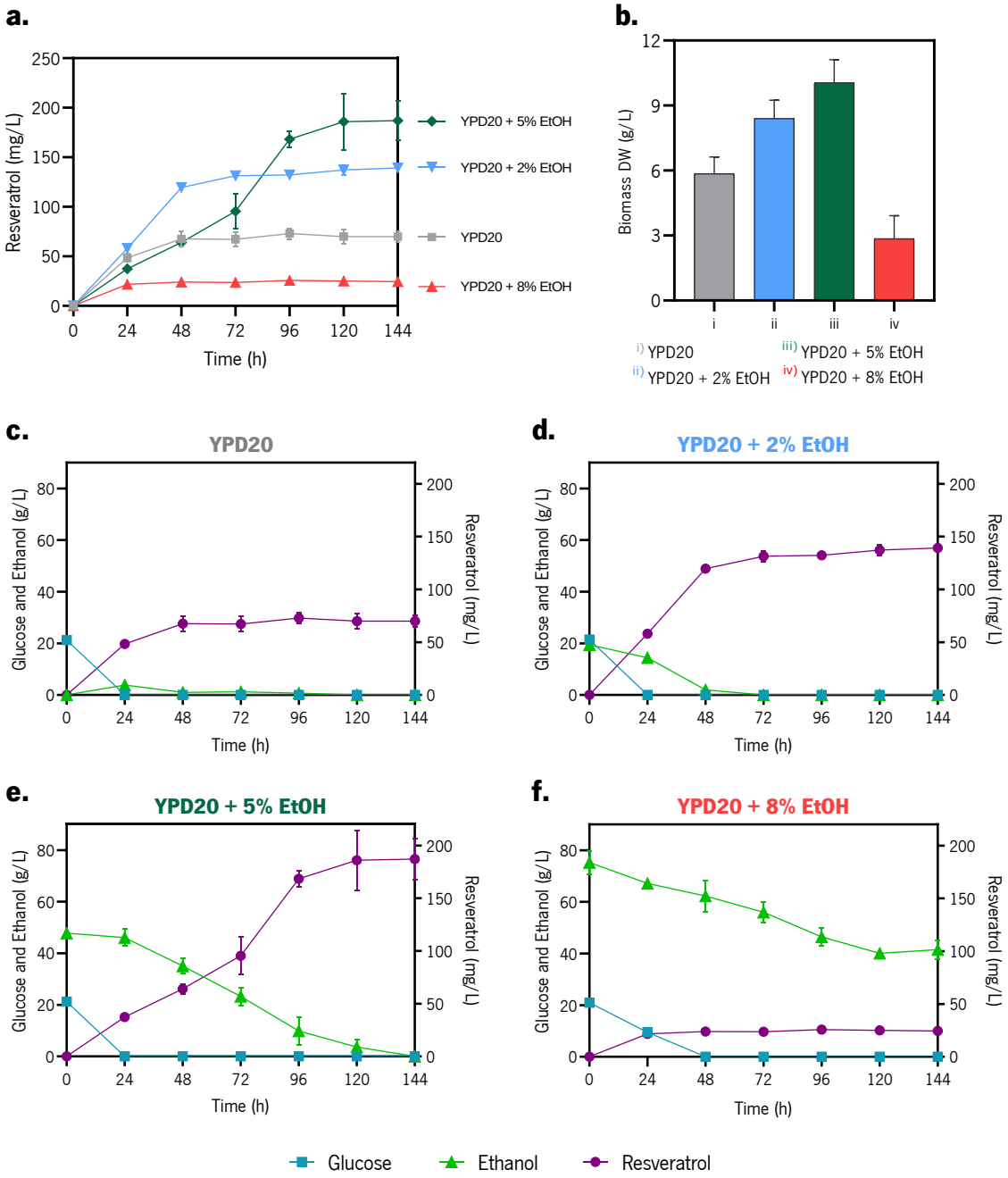

Figure 2.3. Fermentation kinetics and parameters by Ethanol Red RBP strain: **a)** Time course of resveratrol production in YPD20 (grey circles), YPD20 + 2% of ethanol (blue squares), YPD20 + 5% of ethanol (green triangles) and YPD20 + 8% of ethanol (red diamonds). All resveratrol concentrations are statistically different from each other ($p < 0.05$) at the end of fermentation; **b)** Final biomass dry weight (DW) quantification at 144 h of fermentation. Statistical analysis: i vs. ii – ns; i vs. iii*; i vs. iv – ns; ii vs. iii – ns; ii vs. iv*; iii vs. iv**; Time course of glucose (blue squares), ethanol (green triangles) and resveratrol (purple circles) concentrations in YPD20 (**c**), YPD20 + 2% of ethanol (**d**), YPD20 + 5% of ethanol (**e**) and YPD20 + 8% of ethanol (**f**) media. Each data point represents the average ± standard deviation of biological duplicates.

As shown in Figure **2.3**, YPD20 supplementation with 2% of ethanol increased resveratrol production by approximately 2-fold, from 69.88 ± 5.58 mg/L to 139.17 ± 4.33 mg/L, when compared to YPD20, where no ethanol is added. The highest resveratrol titre of 187.07 ± 19.88 mg/L was achieved when 5% of ethanol was supplemented with YPD20 media (Figure **2.3a**). The addition of 8% of ethanol was found to be inhibitory for cell growth, showing reduced biomass formation (Figure **2.3b**), which ultimately resulted in low resveratrol production (24.47 ± 0.25 mg/L), a reduction of nearly 65% compared to no ethanol supplementation. As expected, no ethanol was detected after 24 h of fermentation in YPD20 without ethanol supplementation (Figure **2.3c**), and the increase of ethanol added to the media led to a longer presence of this substrate in the media.[42] While 2% of ethanol added was consumed in the first 48h (Figure **2.3d**), it took 144 h for Ethanol Red RBP to consume all ethanol present in the media when 5% of ethanol was added (Figure **2.3e**). In YPD20 + 8% of ethanol, Ethanol Red RBP was unable to consume nearly half of the ethanol present in the media, with 41.55 ± 3.63 g/L of residual ethanol present in the media (Figure **2.3f**), indicating the clear cell inhibition of this ethanol concentration.

2.3.4. Resveratrol production by SSF of hydrothermally pretreated *Eucalyptus globulus* wood

To evaluate Ethanol Red RBP ability to produce resveratrol from lignocellulosic materials, SSF of the cellulosic fraction of pretreated *Eucalyptus globulus* wood (EGW) was performed. Two different concentrations of solids in the media were assessed. A concentration of 151.65 ± 3.84 mg/L of resveratrol was obtained when 5% of EGW was used, with no residual ethanol and glucose observed at any timepoint after 24 h (Figure **2.4a**). Interestingly, when the percentage of EGW was increased to 10%, doubling the amount the theoretical glucose released to the media, the maximum resveratrol concentration in the media was reduced by 2.4-fold to 63.10 ± 9.72 mg/L (Figure **2.4b**). Theoretically, all non-native compounds produced by engineered *S. cerevisiae* require a net input of ATP for their production from glucose.[37] By having less percentage of solids in the media, when 5% of solids are in suspension (Figure **2.4a**), a smaller amount of glucose is released, therefore favouring respiratory processes. Sugar-limited cultures propel sugar catabolism via respiration,[37] which generates higher amounts of ATP, ultimately favouring resveratrol production. This seems to be also advocated by ethanol accumulation when a higher percentage of solids is present in the media (Figure **2.4b**).

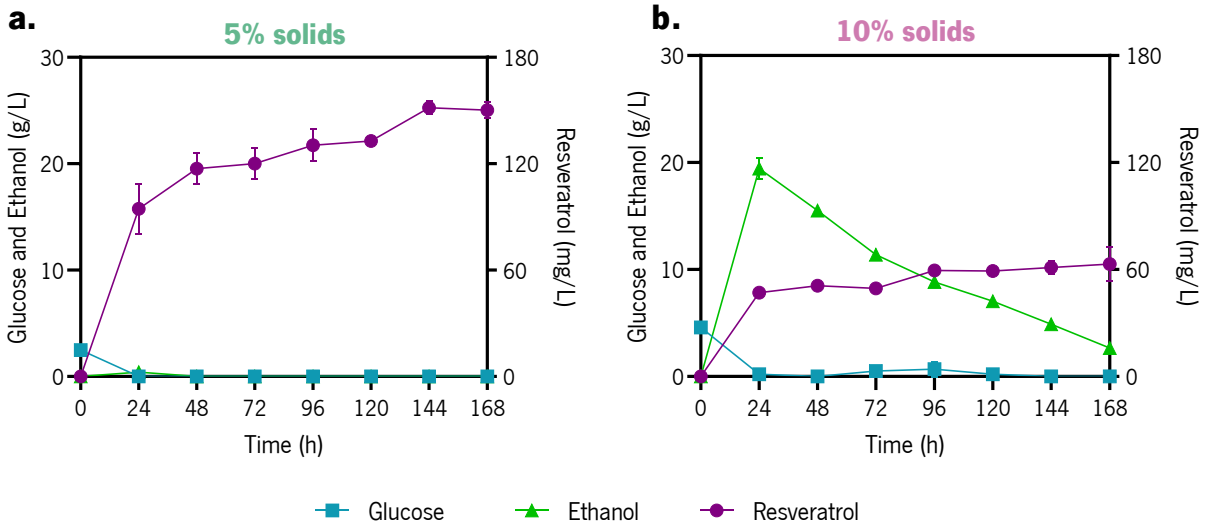

Figure 2.4. Fermentation profiles of resveratrol production from *Eucalyptus globulus* cellulosic fraction by Ethanol Red RBP: **a)** 5% of solids and **b)** 10% of solids. Glucose (blue squares); ethanol (green triangles); resveratrol (purple circles). Each data point represents the average ± standard deviation of biological duplicates. Resveratrol concentration differences between a) and b) are statistically significant (****) at all timepoints after 24 h.

Here, despite no glucose being detected after 24 h, almost 20 g/L of ethanol was produced in the first 24 h, and the strain was not able to consume all ethanol in 168 h (Table **2.2**), even though this ethanol concentration is relatively low (the strain was able to consume this amount of ethanol in 48h in synthetic media – see Figure **2.3**). Fermentation of 5% solids, corresponding to 2.95% of cellulose, showed a yield of 4.14 ± 0.11 mg of resveratrol/g of potential glucose, which is 4.6-fold higher than fermentation with 10% solids (5.9% of cellulose), as shown in Table **2.2**.

Table 2.2. Fermentation parameters of Ethanol Red RBP in *Eucalyptus globulus* cellulosic fraction at 39 °C.

%S	G_{t0} (g/L)	X_{tf} (g/L)	E_{tf} (g/L)	AA_{tf} (g/L)	Gly_{tf} (g/L)	R_{max} (mg/L)	G_{POT} (g/L)	$Y_{R/G}$ (mg/g)	Q_{R24} (mg/L·h)
5	2.50 ± 0.06	0.27 ± 0.18	0.00 ± 0.00	0.36 ± 0.08	0.00 ± 0.00	151.65 ± 3.84	34.13	4.14 ± 0.11	3.94 ± 0.56
10	4.59 ± 0.09	1.30 ± 0.38	2.66 ± 0.58	1.04 ± 0.02	0.71 ± 0.23	63.10 ± 9.72	66.21	0.89 ± 0.14	1.96 ± 0.05

%S, percentage of EGW solids in the medium; G_{t0}, initial glucose concentration; X_{t0}, final xylose concentration; E_{tf}, final ethanol concentration; AA_{tf}, final acetic acid concentration; Gly_{tf}, final glycerol concentration; R_{max}, maximum resveratrol concentration; G_{POT}, theoretical potential glucose; $Y_{R/G}$, yield of resveratrol per glucose; Q_{R24}, productivity of resveratrol at 24 h of fermentation.

Despite being very far from the theoretical yield of 354.7 mg/g,[37] this yield is higher than the yields observed in batch experiments in this study, with higher productivity at 24 h of fermentation (3.94 ± 0.56 mg/L·h). This highlights the advantages of a gradual release of the substrate rather than a traditional high gravity batch fermentation. Moreover, using robust yeast chassis for the introduction of the resveratrol biosynthetic pathway and selecting the top-producing strains enabled resveratrol production under challenging conditions. Only two studies reported resveratrol production by engineered *S. cerevisiae* from carbon sources (glucose and/or ethanol),[8,14] attaining up to 812 mg/L of resveratrol in fed-batch fermentation of glucose, which should not be compared directly to this study. To the best of our knowledge, this is the first report of resveratrol production from lignocellulosic biomass, expanding the possible exploitation of these raw materials for the production of value-added products in a multiproduct biorefinery.

2.4. Conclusions

Here, the aptitude of three different robust industrial yeast strains in comparison with a laboratory strain for resveratrol production from glucose and ethanol at optimal and supra-optimal temperatures was assessed. All industrial strains engineered were able to produce resveratrol, with notable differences between them. At a higher temperature of 39 °C, Ethanol Red RBP showed the best performance. The process configuration here demonstrated has a high potential for improvement. Future studies may focus on boosting resveratrol production by increasing the precursor supply, enhancement of cytochrome P450 activity of the enzyme C4H or the integration of multiple copies of the resveratrol biosynthetic pathway. Evaluation of different fermentation temperatures for a better compromise between the efficiency of saccharification and fermentation can also be addressed. Additionally, fine-tuning of solid loading in the fermentation media and whole slurry SSF can evaluated for improved valorisation of raw materials. Overall, this work establishes grounds for the implementation of an integrated lignocellulose-to-resveratrol process in an industrial context.

2.5. References

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Chapter III.

Resveratrol production for the valorisation of lactose-rich wastes by engineered industrial *Saccharomyces cerevisiae*

This chapter is based on the following original research article:

Costa CE, Romaní A, Teixeira JA, Domingues L. Resveratrol production for the valorisation of lactose-rich wastes by engineered industrial *Saccharomyces cerevisiae*. *Bioresour. Technol.* (In press)

Abstract

Resveratrol is an antioxidant with applications in the food and cosmetic industries. Its biosynthesis can side the hindrances of its extraction from plants. The dairy industry generates tonnes of lactose-rich wastes, which can serve as a carbon source. *Saccharomyces cerevisiae* is an industrial workhorse for biotechnological processes, being unable to naturally metabolise lactose. Here, an *S. cerevisiae* strain was engineered for *de novo* production of resveratrol from lactose. A resveratrol titre of 210 mg/L from 100 g/L of lactose in synthetic media was achieved. Process optimisation increased by 35% the production by a two-stage process, one favouring ethanol production and a subsequent one with stronger agitation favouring ethanol and lactose consumption with conversion into resveratrol. Resveratrol production from cheese whey was further attained. To the best knowledge of the authors, this is the first report on resveratrol production from lactose, relevant in dairy wastes, establishing grounds for future resveratrol-producing lactose-based processes.

Keywords: resveratrol, yeast fermentation, lactose, cheese whey, circular economy

3.1. Introduction

There is a current demand for alternative renewable substrates to produce high-value chemicals. *De novo* production of these compounds from sugars can constitute a valuable and sustainable alternative to processes established on fossil resources.[1] Lactose is a disaccharide present in milk and its derivatives, and the dairy industry generates millions of tons of lactose-rich waste streams.[2] Food waste, in general, is currently a major concern worldwide, with an estimation of 100–170 kg of food waste produced per capita every year.[3] Milk and other processed dairy commodities account for the majority of food waste generated, mainly due to handling, transport or quality issues.[4] Considerable endeavours have been made regarding the management of dairy industry wastes and its by-products (e.g. bioremediation, aerobic treatment or integration in biorefineries), but they are still identified as the most relevant environmental pollutants, containing high amounts of lactose, protein and fat.[5] Lactose is, in fact, the principal constituent of cheese whey powder (CWP). In its turn, CWP is the main by-product of cheese manufacturing processes, having a high content of proteins such as β-lactoglobulin, α-lactalbumin, bovine serum albumin (BSA) and β-casein.[6,7] Lactose and CWP have been widely used for several biotechnological processes, with bioethanol being the most prominent of them.[8]

The yeast *Saccharomyces cerevisiae* is the most common choice for first- and second-generation bioethanol processes,[9] being also used for the production of several chemical targets from different carbon sources, including lignocellulosic materials.[1] Nevertheless, the main drawback of its use in lactose-based processes is that *S. cerevisiae* is not able to naturally metabolise lactose, as it is not able to transport it into the cell nor to hydrolyse lactose into its constituent monomers, glucose and galactose.[10] Several strategies have been applied to *S. cerevisiae* to tackle this obstacle, and one of the most used is the heterologous expression of the *LAC4* and *LAC12* genes, which encode for a β-galactosidase and lactose permease, respectively.[11–13] The expression of these genes under strong constitutive promoters such as *TEF1* and *PGK1* has been recently shown to be valuable for enhanced lactose consumption and high-titre bioethanol production.[14] Furthermore, one path to achieving a sustainable biobased economy could be the establishment of integrated biorefineries that produce biofuels coupled with different value-added chemicals rather than single target production plants.[1]

Resveratrol is a phytoalexin naturally present in plants, having a strong antioxidant activity[15] and a wide range of beneficial properties such as anti-inflammatory,[16] antimicrobial,[17] or antiaging,[18] and many others.[19] Nevertheless, resveratrol has a very short half-life, up to approx. 14 min[20] and particularly low oral bioavailability, which has raised concerns regarding its systemic action.[21] The biological activity of a given compound is highly dependent on its ability to reach its site of action without loss of integrity, being

able to cross over the lipophilic membrane.[6] This constitutes the main drawback when considering plant-derived bioactive molecules, such as resveratrol, for application as therapeutic products, given its low solubility and bioavailability, and its ease of change due to environmental factors like light, temperature, oxygen or pH.[22] One of the approaches often used to tackle this limitation is the encapsulation of bioactive molecules, which protects both the viability and functionality of these compounds.[23] Several materials can be used for encapsulation, and the use of whey protein has been gaining attention in this field, with several studies reporting the use of whey proteins as carrier material for encapsulation of bioactive compounds,[24,25] including resveratrol.[26]

Resveratrol can be extracted from plants or chemically synthesised, but these processes are complex and not sustainable.[27] Alternatively, its *de novo* production from carbon sources can be achieved by the expression of a heterologous pathway that converts tyrosine or phenylalanine into resveratrol.[28,29] Resveratrol production from glucose and ethanol using engineered *S. cerevisiae* strains has previously been reported either in synthetic media[28–30] or using lignocellulosic materials.[30] This process is highly dependent on ATP formation,[31] requiring great amounts of oxygen. Nevertheless, resveratrol yield on ethanol is higher than on glucose.[28–30] The ethanol phase in fermentation using glucose as sole carbon source, where the ethanol produced in the alcoholic fermentation is subsequently converted into resveratrol, has a higher yield than the so-called glucose phase, where ethanol and resveratrol are still being produced simultaneously.[28] Generation of high volumes of ethanol to be subsequently converted into resveratrol would be advantageous as a self-sustainable process configuration, not requiring the addition of an external ethanol supply for improved resveratrol titres. Ethanol fermentation, by its turn, is an anaerobic process, which conflicts with resveratrol production processes. Given this, a fine-tuning between both stages would be beneficial to the overall process. Therefore, a lactose-consuming resveratrol-producing recombinant *S. cerevisiae* strain could comprise two main components: a heterologous pathway to utilise lactose and produce phenylalanine and another one to utilise that phenylalanine for resveratrol production through *p*-coumaric acid (Figure **3.1**). In this study, the development of a strain able to produce resveratrol from lactose was accomplished, further optimising its process configuration for improved yield and titre and application in a waste-valorisation context from cheese whey.

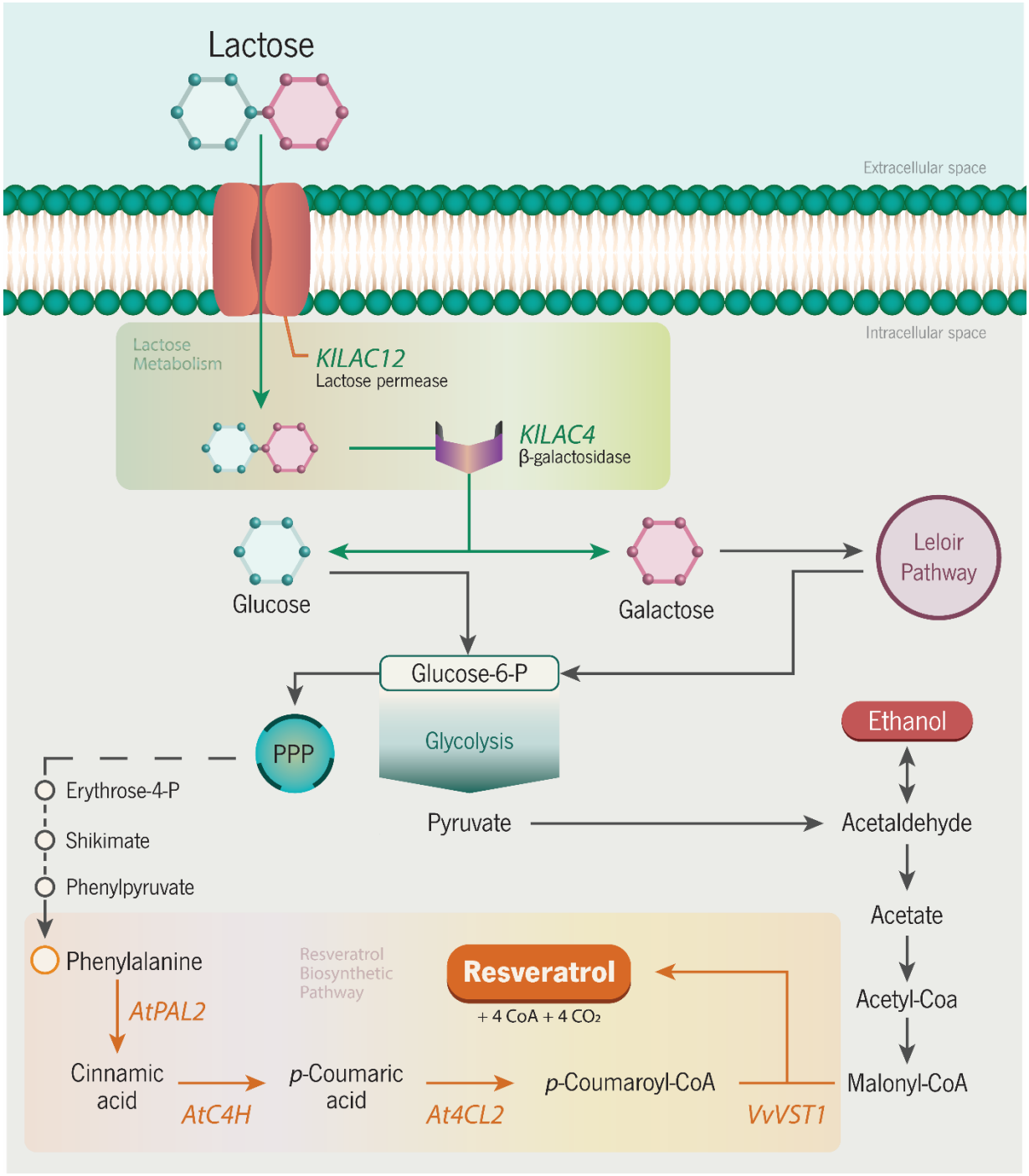

Figure 3.1. Overview of the pathway for resveratrol production using lactose as carbon source. Reactions that enable lactose consumption are represented by green arrows and reactions that convert phenylalanine into resveratrol are represented by orange arrows. *KlLAC12*, lactose permease from *Kluyveromyces lactis*; *KlLAC4*, β-galactosidase from *K. lactis*; *AtPAL2*, phenylalanine ammonia lyase from *A. thaliana*; *AtC4H*, cinnamate-4-hydroxylase from *A. thaliana*; *At4CL2*, 4-coumarate-CoA ligase from *A. thaliana*; *VvVST1*: resveratrol synthase from *Vitis vinifera*; PPP, pentose phosphate pathway; Glucose-6-P, glucose-6-Phosphate; Erythrose-4-P, erythrose 4-Phosphate.

3.2. Materials and methods

3.2.1. Strain

The strain L501 is derived from Ethanol Red®, a commercial *S. cerevisiae* strain developed by *Fermentis, S.I. Lesaffre* for the ethanol industry. Yeast transformations were made following the lithium acetate protocol.[32] The background strain was primarily transformed with a Cas9-expressing plasmid (*KanMX* selection marker, selected on G418).[33] The resulting strain expressing the Cas9 protein was then simultaneously transformed with the desired DNA fragment and the guide RNA (gRNA) plasmid (*NatMX* selection marker, selected on nourseothricin) targeting the chosen insertion site[34] for the insertion of the resveratrol biosynthetic pathway (P_{PGK1}-*4CL2*, P_{TEF1}-*VST2*, P_{TDH3}-*PAL2*, P_{FBA1}-*C4H*) and lactose metabolic pathway (P_{TEF1}-*KILAC4*, P_{PGK1}-*KILAC12*), sequentially. The plasmids used in this study are listed in Table **3.1**.

Table 3.1. List of plasmids used in the study.

Name	Description	Ref.
pCfB2312	Episomal plasmid for Cas9 expression under *TEF1* promoter, *KanMX* selection marker	33
pCfB2909	gRNA helper vector targeting X-4 insertion site, *NatMX* selection marker	34
pCfB3050	gRNA helper vector targeting XII-5 insertion site, *NatMX* selection marker	34
pCfB8531	MarkerFree plasmid, X-4, P_{PGK1}-*4CL2*, P_{TEF1}-*VST2*, P_{TDH3}-*PAL2*, P_{FBA1}-*C4H*	30
B446	MarkerFree plasmid, XII-5, P_{TEF1}-*KILAC4*, P_{PGK1}-*KILAC12*	14

3.2.2. Media and cultivations

The recombinant strain L501 was kept in YPL plates (10 g/L yeast extract, 20 g/L peptone, 20 g/L lactose, 15 g/L agar). For all the experiments in this study, two sequential pre-inocula were made in YP medium with 2% lactose (YPL20). The first was inoculated from a fresh plate and incubated for 24 h. Then, 10% of this pre-culture was transferred to fresh YPL20 media and incubated overnight. Both pre-inocula were made at 30 °C, 300 rpm, in 250 mL baffled shake flasks, with a working volume of 50 mL. Cells from the second pre-inoculum were harvested at 4000 rpm for 5 min, washed with sterile deionised water and resuspended in 0.9% (w/v) sodium chloride to a concentration of 300 g/L of cells (fresh weight, FW). The initial inoculum was 8 g/L of cells (FW) for all fermentation assays.

3.2.3. Fermentation conditions

All fermentations were carried out in 250 mL baffled shake flasks with a working volume of 50 mL. Experiments in synthetic media were made using YPL media: YP (20 g/L peptone, 10 g/L yeast extract) with increasing concentrations of lactose (from 20 g/L to 200 g/L, named YPL20 to YPL200, respectively), at 30 °C, 300 rpm. The experiment to assess the influence of media oxygenation in resveratrol production (Section 3.3.3) was done in YPL media with 100 g/L of lactose at 30 °C, and agitation was set to three different conditions: Scenario S1) 120 rpm; Scenario S2) 300 rpm; Scenario S3) 120 rpm for the first 24 h and 300 rpm afterwards until the end of fermentation. The CWP experiment was carried out using partially deproteinised CWP (pdCWP) with 100 g/L of lactose supplemented with 10 g/L of yeast extract and 20 g/L of peptone, at 30 °C, and agitation was set to 120 rpm for the first 24 h and increased to 300 rpm afterwards until the end of fermentation. All synthetic media were autoclaved at 121 °C for 15 min, while pdCWP was pasteurised at 60 °C for 1 h, after partial protein precipitation.

3.2.4. Cheese whey powder

Cheese whey powder (CWP) was kindly provided by Lactogal (Porto, Portugal). Lactose content in CWP was directly analysed by HPLC, with 0.71 g of lactose present per 1 g of CWP. CWP was sterilised by pasteurisation at 60 °C for 1 hour, followed by 30 min under UV light. CWP fermentation was done by dissolving the desired amount of CWP in deionised water. Partially deproteinised CWP (pdCWP) was obtained by incubation at 90 °C for 15 min, followed by centrifugation at 10000 rpm for 10 min, and subsequent pasteurisation at 60 °C for 1 hour, followed by 30 min under UV light. Protein concentration was determined following the Bradford method,[35] with bovine serum albumin (BSA) as a standard.

3.2.5. Analytical methods

Samples collected from fermentation runs were analysed for lactose and ethanol concentrations by HPLC using a BioRad Aminex HPX-87H column at 60 °C, with a mobile phase of 5 mM H_2SO_4 and a flow rate of 0.6 mL/min. The peaks were detected using a Knauer-IR intelligent refractive index detector. Resveratrol and *p*-coumaric acid were quantified by UHPLC equipped with a Discovery® HS F5 150 mm × 2.1 mm column (particle size 3 mm). The eluent flow rate was 0.7 mL/min. A linear gradient from 5% to 60% of acetonitrile over 10 mM ammonium formate (pH 3.0, adjusted by formic acid) from 0.5–9.5 min was used. Both resveratrol and *p*-coumaric acid were detected by absorbance at 333 nm, with

retention times of 7.1 min and 6.0 min, respectively. Biomass dry weight (BDW) quantification was made by collecting 1 mL of fermentation broth in previously dried and weighed tubes, washed out twice in ethanol and deionised water, sequentially, and incubated at 105 °C for 24 h and weighed again.

3.2.6. Determination of fermentation parameters

Resveratrol yield on lactose ($Y_{R/L}$) was set as the ratio between the maximum concentration of resveratrol produced and total lactose consumed in the fermentation (mg of resveratrol per g of lactose). Resveratrol yield on biomass dry weight ($Y_{R/BDW}$) is the ratio between the maximum concentration of resveratrol produced and biomass concentration at the end of fermentation, in dry weight (mg of resveratrol per g of biomass in dry weight). Maximum lactose consumption rate (L_{CR}) is the steepest slope in lactose concentration curves between two points in each fermentation profile, corresponding to the fermentation period where most lactose was consumed per hour (grams per litre per hour). Resveratrol productivity (Q_R) was defined as the ratio between the maximum concentration of resveratrol produced and the fermentation time at which that concentration was achieved (mg of resveratrol per hour).

3.2.7. Statistical analysis

Variations between the strain profiles in terms of sugar and ethanol concentrations, biomass formation and resveratrol production were tested by repeated measures two-way ANOVA, followed by Tukey post hoc test using GraphPad Prism for Windows version 8.02. Statistical significance was established at *p*-value < 0.05 for the comparisons and marked by “ns” (non-significant) - $p > 0.05$; * - $p < 0.05$; ** - $p < 0.01$; *** - $p < 0.001$; **** - $p < 0.0001$.

3.3. Results and discussion

3.3.1. Construction of a lactose-consuming resveratrol-producing yeast strain

Heterologous expression of *LAC4* under control of *TEF1* and *LAC12* under control of *PGK1* was made into a previously engineered resveratrol-producing Ethanol Red strain (Figure **3.2a**).[30] Ethanol Red is an industrial diploid strain developed for ethanol fermentation capable to cope with high sugar concentrations (Lesafre Advanced Fermentations), being able to simultaneously consume glucose and galactose,[36] a valuable feature for effective lactose metabolisation. This strategy was selected due to previous findings that showed that a stronger constitutive promoter on *LAC4* increases the yeast capacity to metabolise higher lactose content with steadier lactose uptake.[14] This leads to gradual sugar consumption and ethanol production, which may benefit resveratrol production over ethanol accumulation. The resultant strain, L501, showed very good lactose metabolisation capability, being able to consume all lactose in the media (approx. 21.5 g/L) in 12 hours. The recombinant strain produced over 42 mg/L of resveratrol exclusively from lactose (Figure **3.2b**), which to the best knowledge of the authors is the first report of resveratrol production from lactose.

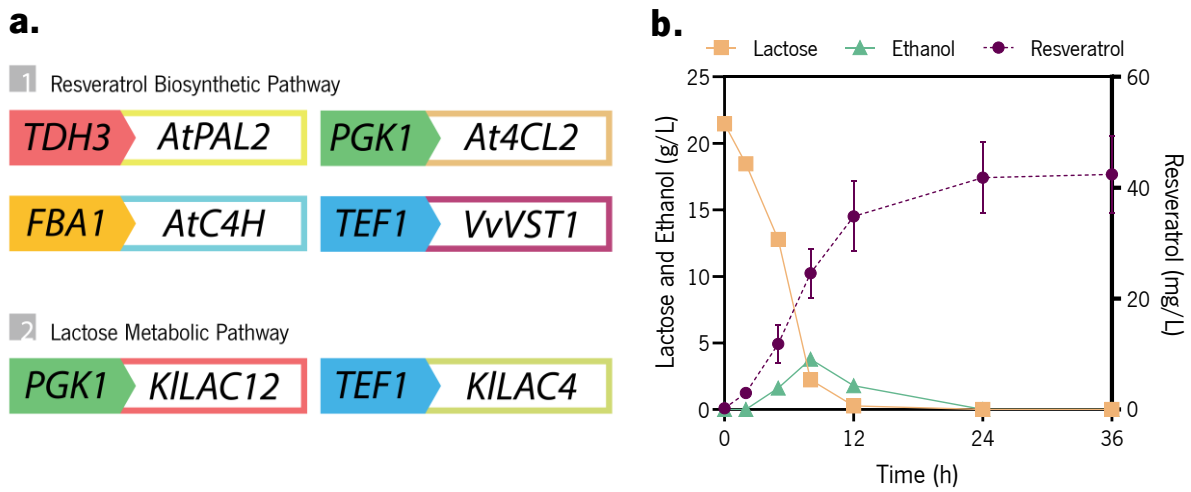

Figure 3.2. a) Schematic illustration of the genotype of the strain L501. Initially, (1) Resveratrol Biosynthetic Pathway was expressed in an Ethanol Red strain[30] and, subsequently, (2) the Lactose Metabolic Pathway was introduced in the strain, generating the strain L501; b) Time course of resveratrol production from medium containing lactose as sole carbon source.

3.3.2. Characterisation of resveratrol production in synthetic media

As shown in Figure **3.2**, L501 can efficiently produce resveratrol from lactose. In this sense, this strain was further characterised in synthetic media with increasing lactose concentration, from 50 g/L to 200 g/L (Figure **3.3**). These concentrations were selected taking into account that cheese whey and whey permeate are among the major industrial waste streams containing approx. 50 g/L of lactose, which can be concentrated up to 200 g/L, near lactose solubility in water. Up to 100 g/L of lactose in the fermentation, resveratrol yield on lactose remains approximately constant, ranging between 1.98 to 2.03 mg of resveratrol per gram of lactose consumed (Figure **3.3a, 3.3b**). In YPL100 media, the strain L501 is able to produce 210 mg/L of resveratrol, the highest titre obtained (Figure **3.3b**). An increase in lactose concentration to 150 g/L and 200 g/L of lactose did not improve resveratrol titres, producing 187 mg/L and 185 mg/L, respectively (Figure 3c, 3d). This led to a consequent decrease in resveratrol yield on lactose to 1.20 mg/g for YPL150 fermentation and 0.89 mg/g in YPL200.

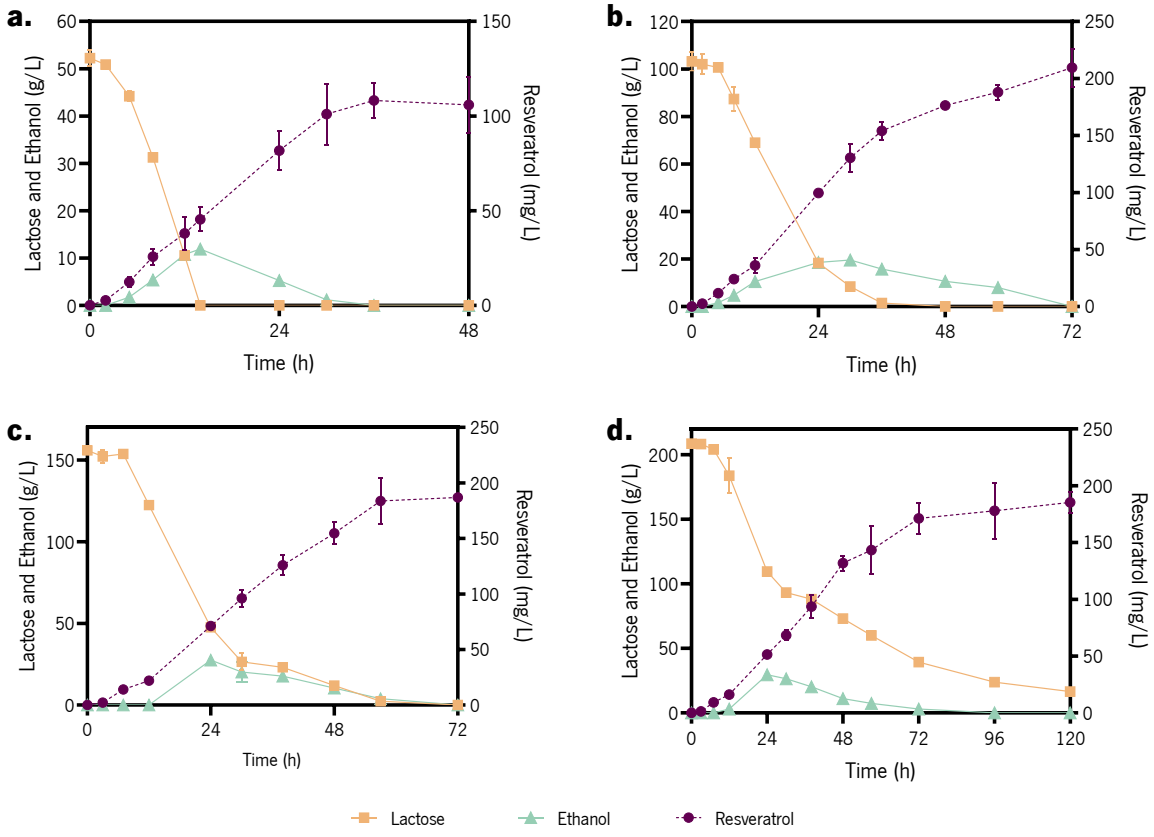

Figure 3.3. Fermentation profiles for characterisation of L501 strain in synthetic media with increasing concentrations of lactose: **a)** 50 g/L of lactose; **b)** 100 g/L of lactose; **c)** 150 g/L of lactose; **d)** 200 g/L of lactose.

The strain L501 showed an excellent aptitude to metabolise lactose up to 150 g/L, consuming all the lactose in the fermentation media in 72 hours or less. In YPL200 media, the strain consumed 92% of the lactose in the media, with a residual lactose accumulation of 16.5 g/L. The lactose-consuming strain without the expression of the resveratrol pathway was previously shown to have optimal lactose consumption for very high gravity ethanol fermentation processes, consuming all the lactose in fermentation media at 200 g/L, even though at this concentration lactose uptake rate was considerably reduced when compared to the lower initial lactose content.[14] Here, additional expression of the resveratrol biosynthetic pathway might further challenge lactose assimilation by increasing the metabolic burden, which may help to explain the difficulty of the strain to consume all lactose in the media, which by its turn caps the overall resveratrol titres. The relatively low resveratrol titres observed in batch fermentation (mainly in the 100-200 mg/L range) might be explained by the high energy demand of resveratrol biosynthesis, which is highly dependent on ATP and biomass formation, as demonstrated by Vos et al.[31] Additionally, the authors demonstrated that up to 27% of the glucose fed to the media was used for cellular maintenance energy requirement, rather than channelled towards growth or resveratrol production, especially at lower growth rates,[31] which could help to explain the modest titres (below 0.5 g/L) obtained in batch fermentation in all studies throughout the literature.[28–30] Nevertheless, when compared with a previous study using the same yeast background and identical resveratrol pathway from glucose and ethanol,[30] in equivalent fermentation conditions, the maximum titre obtained from 100 g/L of lactose was higher [210 mg/L against 187 mg/L from 20 g/L of glucose supplemented with 5% of ethanol (w/v)], which further highlights the relevance of the use of lactose as a carbon source for resveratrol production.

Figure **3.3** also shows that lactose consumption rate is slowed down when ethanol starts to be consumed, being especially noticeable in higher lactose concentrations. Between 12 h and 24 h of fermentation, the lactose consumption rate in YPL200 is around 6.2 g/L·h, but this decreases to approx. 1.4 g/L·h between 38 h and 72 h of fermentation, and to 0.5 g/L·h from 72 h until the end of fermentation (120 h). This is also clearly observed in the fermentation of 150 g/L of lactose, where lactose consumption rate drops from its maximum of 6.2 g/L·h between 7 h and 24 h of fermentation to only 0.9 g/L·h between 30 h and 57 h of fermentation, which coincides with the beginning of ethanol consumption. Interestingly, the maximum ethanol consumption rate of fermentations with more initial lactose concentrations (150 and 200 g/L) is higher (6.2 g/L·h for both) than fermentations with 50 g/L or 100 g/L of initial lactose (5.2 and 4.5 g/L·h, respectively) (Table **3.2**). The heterologous lactose consumption route expressed in this strain has *LAC4*, encoding the β-galactosidase responsible for

lactose hydrolysis, under the control of *TEF1*, which is a stronger promoter than *PGK1*, the promoter used with *LAC12*, the lactose permease.[37] The lactose consumption rate is higher in fermentations with higher lactose concentrations probably due to the high activity of the β-galactosidase associated with high lactose availability. Resveratrol is a phytoalexin produced in plants in response to stresses such as fungal infection,[38] having antimicrobial activity.[17] Therefore, the possible inhibition of cell growth associated with high resveratrol concentrations in lactose was also considered. Even though resveratrol minimum inhibitory concentration in *S. cerevisiae* has been previously reported to be around 10 to 20 mg/L,[39] this is highly dependent on the yeast strain chassis. No considerable inhibition of cell growth in lactose or glucose was observed in the strain L501 up to 0.5 g/L of resveratrol in the fermentation media, either in plate cultures or liquid media cultivations (data not shown). This also highlights the value of choosing industrial robust yeast strains such as Ethanol Red for resveratrol biosynthesis. Data from biomass formation at the end of fermentation appears to follow this trend as well, with a statistically significant (*p*-value < 0.0001) increase in biomass dry weight up to 100 g/L, which stabilises above that lactose concentration, with no statistically significant differences observed between 100 and 200 g/L of initial lactose in the fermentation media (*p*-value > 0.05). Fermentation of 100 g/L of initial lactose, therefore, exhibited a higher yield of resveratrol on biomass, allied to a higher titre and yield on lactose (Table **3.2**). Given this data, this initial lactose concentration was set as the target for subsequent experiments to maximise the yield of batch fermentation.

Table 3.2. Fermentation parameters of lactose synthetic media (YPL) fermentations using the strain L501.

ID	L_0 (g/L)	R_{max} (mg/L)	Q_R (mg/L·h)	$Y_{R/L}$ (mg/g)	L_{CR} (g/L·h)	L_{final} (g/L)	BDW (g/L)	$Y_{R/BDW}$ (mg/g)
YPL20	21.5 ± 0.3	42 ± 7	1.40 ± 0.21	1.97 ± 0.30	2.6 ± 0.1	0.0 ± 0.0	8.7 ± 0.2	4.90 ± 0.87
YPL50	52.2 ± 1.8	108 ± 10	3.01 ± 0.26	2.07 ± 0.11	5.2 ± 0.1	0.0 ± 0.0	12.6 ± 0.2	8.56 ± 0.70
YPL100	103.2 ± 3.9	210 ± 17	2.91 ± 0.23	2.03 ± 0.09	4.5 ± 1.1	0.0 ± 0.0	16.0 ± 1.0	12.87 ± 0.10
YPL150	155.9 ± 2.7	187 ± 2	2.60 ± 0.02	1.20 ± 0.01	6.2 ± 0.1	0.0 ± 0.0	15.9 ± 0.7	11.91 ± 0.32
YPL200	208.5 ± 3.6	185 ± 9	1.54 ±0.08	0.89 ± 0.03	6.2 ± 1.1	16.5 ± 2.5	17.5 ± 0.1	10.59 ± 0.45

L_0 is the initial lactose present in the fermentation media at time = 0 h; R_{max} is the maximum resveratrol concentration at the end of fermentation; Q_R is the productivity of resveratrol; $Y_{R/L}$ is the yield of resveratrol on lactose; L_{CR} is the maximum lactose consumption rate; L_{final} is the residual lactose concentration at the end of fermentation; BDW is the concentration of biomass in dry weight at the end of fermentation; $Y_{R/BDW}$ is the yield of resveratrol on biomass (dry weight)

3.3.3. Influence of media oxygenation in resveratrol production

Resveratrol is highly dependent on ATP formation,[31] therefore, oxygenation of the fermentation media is key for resveratrol production. In batch flask fermentation, this is achieved by controlling the agitation of the flasks. Nevertheless, the ethanol stage in resveratrol production has been previously shown to have higher yields than the glucose stage.[28–30] Ethanol fermentation is an anaerobic process, which requires less agitation in the fermentation media. Therefore, a fine-tuning of the aeration of the fermentation media might be optimal for resveratrol production, dividing the fermentation into a stage favouring ethanol and another one where the ethanol produced in the fermentation is used to produce resveratrol. To test this hypothesis, agitation of the shake flasks was set to three different scenarios: one with low agitation, at 120 rpm, favouring ethanol production (S1); another with high agitation, at 300 rpm, favouring only media oxygenation (S2); and a final one with low agitation (120 rpm) for the first 24 hours, and 300 rpm after that time point, favouring resveratrol production (S3).

Results in Figure **3.4** show that, after 24 h, fermentation at 300 rpm had already consumed nearly all the lactose in the fermentation media (approx. 100 g/L), producing 21.8 g/L of ethanol and 113 mg/L of resveratrol. On the other hand, in the same timeframe, both S1 and S3 scenarios (120 rpm for the first 24 h) produced only approx. 12 mg/L of resveratrol and between 11 and 12 g/L of ethanol but used only approx. 32 g/L of lactose. The biggest differences between the three strategies occurred between 24 h and the end of fermentation (96 h). Scenario S1, where agitation was kept at 120 rpm for the entire fermentation, achieved a final resveratrol titre of only 33 mg/L. The maximum ethanol concentration of 23.6 g/L was achieved at 38 h and after that time, less than 2 g/L of ethanol was consumed until 96 h of fermentation, also showing 24.9 g/L of residual lactose. This is according to what was expected, as low agitation led to poor media oxygenation, which does not favour resveratrol production and, in this sense, reduced consumption of ethanol is observed. On the other hand, no residual lactose or ethanol was observed in scenario S2, which showed a maximum production of 195 mg/L of resveratrol, being in accordance with the data in Figure **3.3b**, where this strain showed no difficulties to ferment 100 g/L of lactose. Increasing the agitation of the media, and consequently its oxygenation, after an initial period of 24 h at 120 rpm, was revealed to be the best strategy for increased resveratrol titres among the three evaluated. In scenario S3, a resveratrol titre of 284 mg/L of resveratrol was attained, a 1.46-fold increase compared to scenario S2, and an 8.52-fold increase compared to scenario S1. Scenario S3 clearly showed an initial stage (first 24 h) favouring ethanol production, and a second one, after 24 h, favouring resveratrol production. In S3, ethanol produced in the first 24 h is almost immediately consumed after the increase in agitation, achieving a maximum ethanol concentration of 13.6 g/L at 28 h of fermentation.

The first 24 h at low agitation does not seem to interfere with the overall yeast cell growth, as the final biomass in dry weight in scenarios S2 and S3 show no statistically significant differences (approx. 17 g/L), nearly doubling the cell growth achieved in scenario S1 (8.8 g/L of biomass DW), which also shows the dependence on cell growth and ATP formation of resveratrol production.[31] Therefore, this process configuration was used for further fermentations.

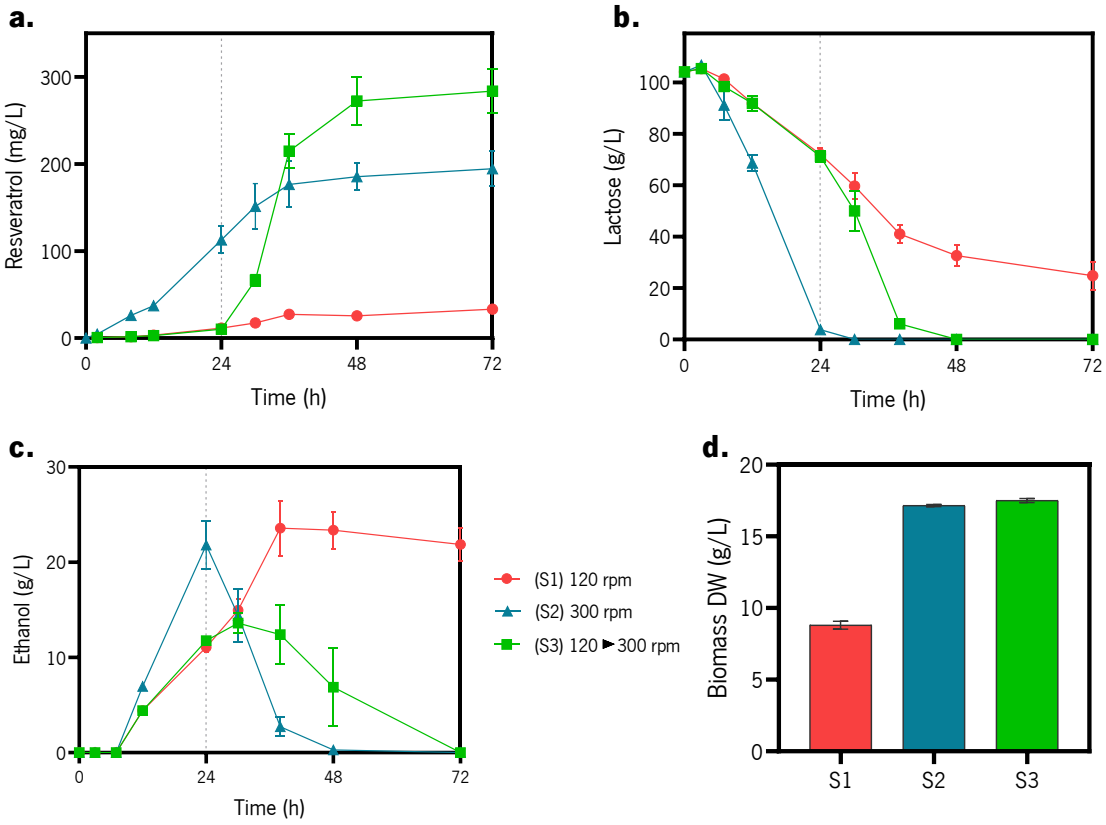

Figure 3.4. Fermentation profiles and parameters from L501 strain in synthetic media with 100 g/L of lactose in 3 scenarios: (Scenario S1) 120 rpm; (Scenario S2) 300 rpm; (Scenario S3) 120 ▶ 300 rpm, after 24 h of fermentation. **a)** Resveratrol profile; **b)** Lactose profile; **c)** Ethanol profile; **d)** Biomass dry weight quantification. Vertical dashed arrows signalise the increase of 120 rpm to 300 rpm in scenario (S3). Statical analysis at the end of fermentation: a) A vs B: ***; A vs C: ***; B vs C: **; b) A vs B: **; A vs C: **; B vs C: ns; c) A vs B: ***; A vs C: ***; B vs C: ns; d) A vs B: ****; A vs C: ****; B vs C: ns

3.3.4. Resveratrol production from cheese whey

Based on the previous results assessing the impact of aeration conditions on resveratrol production, cheese whey powder was evaluated as a source of lactose using the same strategy. An initial lactose concentration of 99.1 g/L of lactose was found in approx. 142 g/L of CWP. Unexpectedly, the initial fermentation of CWP showed reduced resveratrol production of 17 mg/L (data not shown). Resveratrol is

known to interact with β-casein, BSA and β-lactoglobulin, present in cheese whey.[6] Also, precipitation of CWP proteins in the presence of ethanol has already been reported[40] and, in addition to the ethanol produced during the fermentation, the addition of absolute ethanol in equal volume to the sample is necessary to dissolve resveratrol for quantification. This would explain the low resveratrol titre detected even though no residual lactose or ethanol were found at the end of fermentation. Resveratrol is possibly precipitating bonded together with CWP proteins and only a small fraction of it remains in the supernatant used for quantification.

To evaluate this hypothesis, partial precipitation of the CWP proteins was accomplished (Figure **3.5a**), removing approx. 52% of its total protein content, in order to see if resveratrol production would increase with reduced protein content in the fermentation of partially deproteinised CWP (pdCWP). After the first 24 h, and differently than the observed in synthetic media (Figure **3.4b**), approx. 65% of the lactose was consumed in the low agitation stage, producing 28.0 g/L of ethanol but only 13 mg/L of resveratrol (Figure **3.5b**). It appears that pdCWP fermentation favours ethanol production, with increased lactose consumption compared to synthetic media, as resveratrol production at this time point was nearly the same as in YPL100. After 24 h, agitation was increased to 300 rpm. In the following 48 h, resveratrol concentration increased up to 111 mg/L (6.6-fold higher compared to CWP). This appears to be in accordance with the abovementioned hypothesis that low resveratrol titres in CWP fermentation are a question of detection rather than production, suggesting that indeed resveratrol is precipitating together with CWP proteins. The use of whey permeate, a lactose-rich stream produced in parallel with cheese whey protein concentrate, instead of CWP could be an interesting alternative to side this hinder. The permeate preserves most of the lactose content of CWP while having reduced protein content, it is generated in high amounts and thus, its use as the substrate would contribute to solving the permeate disposal environmental problem.

On the other hand, researchers have previously recurred to encapsulation of plant polyphenols using CWP proteins as carrier molecules to increase bioavailability in the oral delivery of these compounds.[41] Therefore, one interesting course for the valorisation of cheese whey powder for resveratrol production might not be to isolate resveratrol from CWP proteins but rather to produce it directly for increased bioavailability, taking advantage of the bond between them.

In this study, an Ethanol Red strain was used as chassis for the genetic modifications as it is able to co-consume glucose and galactose[36] which impacts the engineered lactose metabolisation capacity.[14] In addition, it has a very robust background, with increased value in the framework of lignocellulosic processes, due to its ability to cope with fermentation inhibitors[36,42–44] and high temperatures.[45–47] Indeed,

resveratrol production from lignocellulosic materials and at temperatures up to 39 °C was previously shown using the Ethanol Red chassis engineered with the resveratrol pathway.[30] These features might have increased significance when considering multi-waste strategies for the valorisation of residues, such as a mixture of lignocellulosic materials with cheese whey, which have been previously shown to be a valuable strategy for ethanol production.[36,48,49] In this sense, the strain L501 can be attractive for integration in future strategies for the simultaneous valorisation of both cheese whey or dairy waste streams and lignocellulosic biomass.

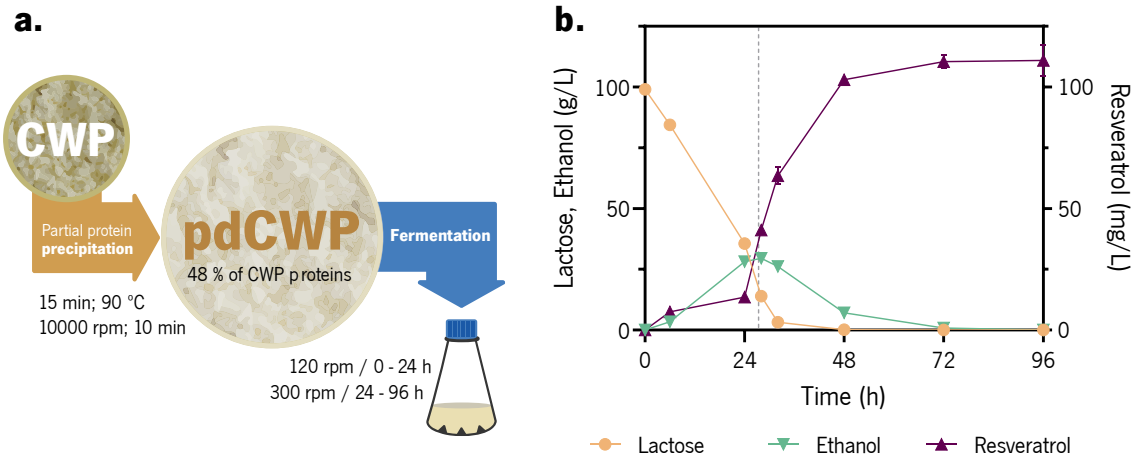

Figure 3.5. a) Schematic representation of CWP processing and fermentation; **b)** Fermentation profiles of L501 strain in cheese whey powder with 100 g/L of initial lactose content. The vertical dashed arrow signalises the increase of 120 rpm to 300 rpm.

Previous studies regarding batch fermentation of engineered *S. cerevisiae* strains expressing solely the resveratrol biosynthetic pathway core genes reported titres below 100 mg/L of resveratrol for glucose fermentation[29,30] and 187 mg/L of resveratrol for simultaneous use of glucose and ethanol as carbon sources.[30] This study shows not only the impact of fine-tuning the aeration of the fermentation media but the benefits of a lactose-based bioprocess for resveratrol production are further highlighted by the increased titres here displayed. As of 2021, the amount of cheese whey generated worldwide is on the verge of surpassing 200 million metric tonnes per year, which roughly represent near 10 million tonnes of lactose wasted on an annual basis. Even though the highest concentration of 284 mg/L of resveratrol achieved from 100 g/L of lactose in this study is very far from the theoretical yield (on glucose, the maximum theoretical yield is estimated to be 354.7 mg/g of sugar[31]), this amount of lactose would be enough to produce more than 28 million kg of resveratrol. The wide range of lactose-rich streams wasted by the dairy industry could also be worth exploring in the future, further aiding in its sustainable disposal.

3.4. Conclusions

For the first time, the feasibility of using lactose as a substrate for resveratrol production was shown. The importance of fine-tuning media oxygenation was demonstrated, where a defined two-stage process with different agitation increased resveratrol titres. Cheese whey can be used as a lactose source for resveratrol bioprocesses, and the exploitation of the interactions between resveratrol and CWP proteins can lead to alternative applications. Other dairy wastes like cheese whey permeate, lacking CWP proteins, would also be interesting to investigate in the future. This study provides valuable insight for future lactose-based processes for resveratrol production, following a circular bioeconomy point-of-view.

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Chapter IV.

Valorisation of wine wastes by *de novo* biosynthesis of resveratrol using a recombinant xylose-consuming industrial *S. cerevisiae*

This chapter is based on the following original research article:

Costa CE, Romaní A, Møller-Hansen I, Teixeira JA, Borodina I, Domingues L. Valorisation of wine wastes by *de novo* biosynthesis of resveratrol using a recombinant xylose-consuming industrial *S. cerevisiae.* (In preparation)

Abstract

Resveratrol is a stilbenoid with strong antioxidant activity and several beneficial properties for human health. Plant extraction of resveratrol from natural sources is expensive and non-sustainable, owing to the low quantity of biomass and generally restricted availability. Biotechnological production of resveratrol can overcome these drawbacks. Here, the heterologous resveratrol biosynthetic pathway (via phenylalanine) was expressed in a xylose-consuming *Saccharomyces cerevisiae* strain. We further elucidated the roles of the pentose phosphate pathway and nutritional supplementation in resveratrol titres. By simultaneous fermentation of glucose and xylose, a 1.31-fold increase in resveratrol titre was observed when compared with glucose-only cultivation at the same carbon molarity, achieving a titre of 388 mg/L. The recombinant strain was able to consume all sugars present in wine wastes, including non-naturally metabolised sugars like xylose. This allowed the valorisation of different vineyard residues, such as wine lees, grape must and hemicellulosic hydrolysate from vine pruning, achieving titres between 167.1 and 282.7 mg/L of resveratrol. The potential of biotechnological processes over conventional processes like plant extraction is emphasised. This is the first report on the use of renewable carbon sources for resveratrol production from xylose and the use of winery by-products as a substrate to produce this stilbenoid. The expanded multi-sugar utilisation capacity of this yeast is valuable in a biorefinery context and obtaining high-value products such as resveratrol is critical to increasing process feasibility following a circular economy concept.

Keywords: resveratrol, yeast fermentation, xylose, wine waste, circular economy

4.1. Introduction

Resveratrol is a stilbenoid with strong antioxidant activity that plays a role in plant defence against environmental stresses.[1] Even though therapeutical effects of resveratrol in humans are still unclear regarding its mode of action and molecular target, several beneficial properties are attributed to resveratrol, such as treatment/prevention of cardiovascular diseases,[2] anti-inflammatory[3] and anti-ageing properties.[4,5] Microbial production of resveratrol from carbon sources can be attained through tyrosine[6] or phenylalanine[7,8] via the shikimate pathway. In the latter, phenylalanine is converted into *p*-coumaric acid through the intermediary cinnamic acid. Coumaric acid is then converted into *p*-Coumaroyl-CoA, which ultimately leads to resveratrol formation by condensation of this precursor with 3 molecules of malonyl-CoA[9] (Figure **4.1**). Resveratrol production has been previously reported in engineered *Saccharomyces cerevisiae* from different carbon sources such as glucose and ethanol.[6–8]

The current environmental and economic challenges have pushed the need for sustainable alternatives to produce energy and value-added products from renewable resources.[10]. In this sense, using agro-industrial wastes has been gaining attention as possible feedstocks for biotechnological processes.[11] The Iberian Peninsula is an exceptional territory to produce a wide variety of wines. Both Portugal and Spain are among the main wine producers on the planet.[12] This extensive manufacture generates large quantities of wastes like vine pruning residues, grape pomace, or wine lees. Wine lees consist of residual fermentative yeast and other particles, and have high nitrogen and organic content, including acids, phenols, and ethanol.[13] They have been previously proposed as low-cost nutrients for microbial production of biochemical products,[14] but could also be used as substrate due to their ethanol content. Additionally, there are substantial volumes of grape must, rich in glucose and fructose, that are not used for wine production, due to being a surplus or having low quality. Grape must can be an interesting carbon source for biotechnological processes for value-added products generation, being already reported for the production of erythritol or mannitol.[14,15] Furthermore, vine pruning, which consists of the necessary removal of parts of a grapevine to renew its canes, generates significant amounts of lignocellulosic biomass, being the major by-product of viticulture, and both its cellulosic and hemicellulosic fractions can be valuable substrates for biotechnological processes.[16] To attain fermentable sugars from vine pruning, pretreatment and hydrolysis are mandatory steps to break down the recalcitrant and typical structure of lignocellulosic biomasses. Hydrothermal treatment (also known as autohydrolysis) of vine pruning has been employed as the first step of a biorefinery for the solubilisation of hemicellulose into xylooligosaccharides[17] and for ethanol production from cellulose.[18] The pentose sugar xylose is the most abundant sugar in the hemicellulosic fraction of vine pruning, followed by glucose. Xylose can comprise

up to 20% of the total carbon content of lignocellulose.[19] However, it is not naturally metabolised by *S. cerevisiae*, an industry workhorse for ethanol fermentation.[20]

Xylose metabolism in *S. cerevisiae* can be achieved by two different pathways, enabling the conversion of xylose into xylulose. The oxidoreductase pathway involves a two-step reaction: xylose reductase (XR) reduces xylose to xylitol, which is then oxidised to xylulose through the action of xylitol dehydrogenase (XDH). These two pathways can act independently or in concert.[21] A cofactor imbalance between the predominantly NADPH-dependent XR and the NAD+-dependent XDH leads to xylitol accumulation, thus capping the carbon flow to other metabolic routes such as ethanol or other value-added compounds production, consequently reducing fermentation yields.[22] Furthermore, an unspecific aldose reductase encoded by the endogenous *GRE3* gene can also convert xylose to xylitol, thus intensifying xylitol build-up.[23] Several studies have relied on the deletion of the *GRE3* gene to minimise xylitol accumulation,[24,25] while its overexpression has led to a xylitol high-production phenotype[26–28]. The isomerase pathway relies on a single reaction catalysed by xylose isomerase (XI), which can convert xylose directly into xylulose without requiring any cofactor.[29,30] Xylulose is then phosphorylated by xylulokinase (XK) to xylulose-5-phosphate (X5P) and shuffled to glycolysis via the pentose phosphate pathway (PPP). Several studies focused on the overexpression of XK, either the native *S. cerevisiae XKS1*[24,25,31] or the *S. stipitis XYL3,*[32] as well as the non-oxidative PPP genes (*RPE1*, *RKI1*, *TKL1*, *TAL1*) to enhance xylose assimilation.[24,25,30,33–35]

Besides its central role in xylose metabolism, the PPP is also vital to producing several valuable compounds like polyols, biofuels or phenylpropanoids. The PPP is tightly related to several metabolic steps of glycolysis, either by the assimilation of glucose-6-phosphate in the oxidative part of the PPP or the reversible reactions of the non-oxidative PPP to form fructose-6-phosphate or glyceraldehyde-3-phosphate.[36] Furthermore, the non-oxidative PPP is responsible for generating erythrose 4-phosphate (E4P) in the cell. E4P with phosphoenolpyruvate (PEP) from glycolysis condensate to form 3-deoxy-D-arabinoheptulosonate-7-phosphate (DAHP), which flows into the shikimate pathway for the biosynthesis of aromatic amino acids like tyrosine and phenylalanine (Figure **4.1**). Malonyl-CoA is also an essential precursor to producing resveratrol. Furthermore, because malonyl-CoA is primarily employed as an important intermediary in fatty acid biosynthesis to maintain cell development, only a small amount of malonyl-CoA is available for resveratrol biosynthesis, which is a significant hurdle in resveratrol production.[37] Malonyl-CoA is converted from acetyl-CoA in a reaction catalysed by acetyl-CoA carboxylase (encoded by *ACC1*), which originated in the yeast cell from acetate. In its turn, acetate is derived from acetaldehyde that results from pyruvate or ethanol consumption, which can help to balance the supply of malonyl-CoA in the yeast cell (Figure **4.1**).

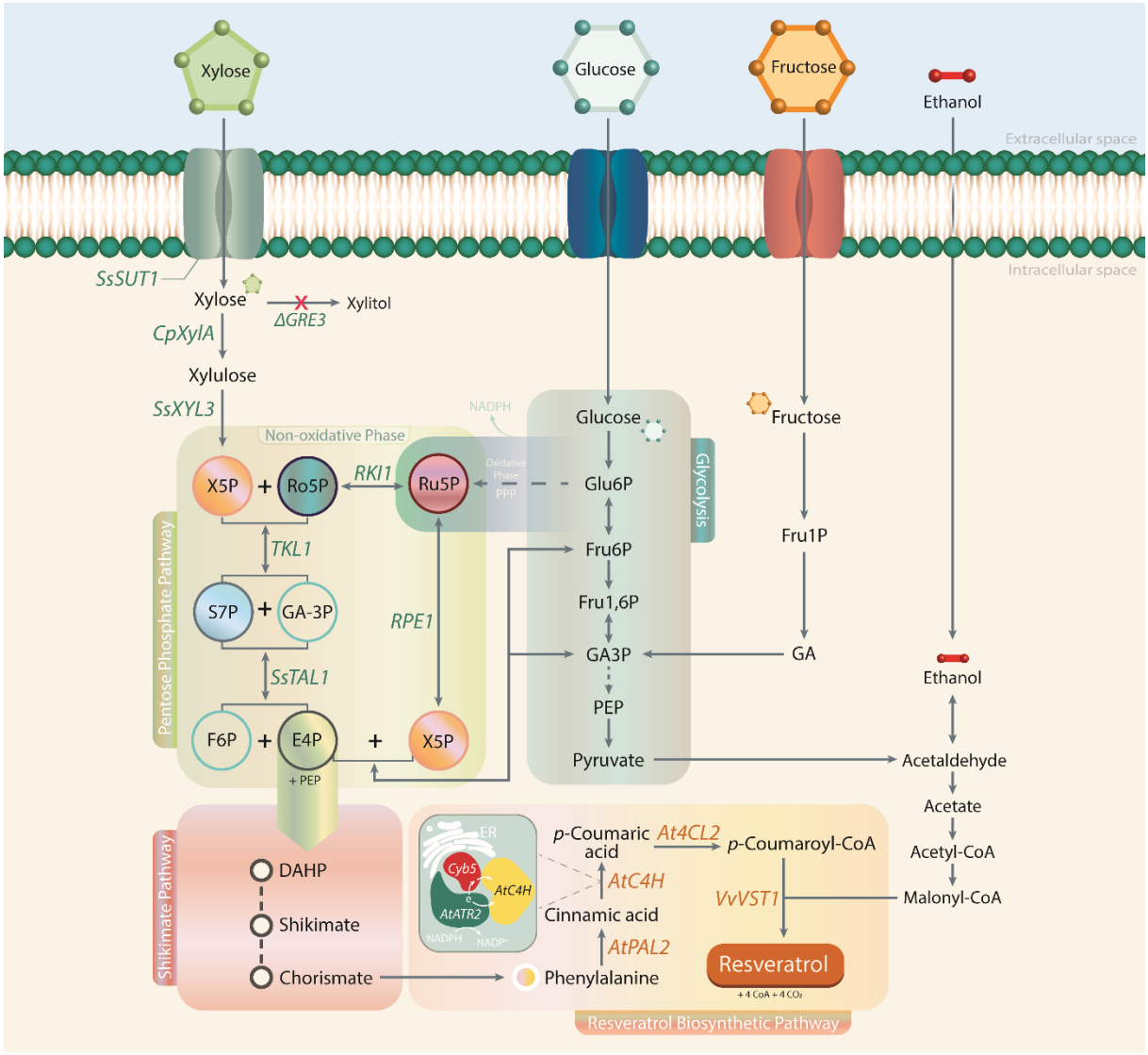

Figure 4.1. Metabolic pathway for resveratrol production from several carbon sources. Single arrows represent single reaction steps and dashed arrows represent multiple reaction steps. Genes (over)expressed in the strain L543 are represented next to the reactions that are catalysed by them. *SsSUT1* encodes for a sugar transporter with a higher affinity for xylose from *S. stipitis*; *CpXylA*, xylose isomerase from *Clostridium phytofermentans*; *SsXYL3*, D-xylulokinase from *S. stipitis*. Glycolysis: Glu6P, glucose 6-phosphate; Fru6P, fructose 6-phosphate; Fru1,6P, fructose 1,6-biphosphate; GA3P, glyceraldehyde 3-phosphate; PEP, phosphoenolpyruvate. Fru1P, fructose 1-phosphate; GA, glyceraldehyde. Pentose Phosphate Pathway: X5P, xylulose 5-phosphate; Ro5P, ribose 5-phosphate; Ru5P, ribulose 5-phosphate; S7P, sedoheptulose 7-phosphate; E4P, erythrose 4-phosphate; *RPE1*, D-ribulose 5-phosphate 3-epimerase; *RKI1*, D-ribose-5-phosphate ketol-isomerase; *TKL1*, transketolase; *SsTAL1*, transaldolase from *S. stipitis*. DAHP, 3-deoxy-D-arabinoheptulosonate 7-phosphate Resveratrol Biosynthetic Pathway: AtPAL2, phenylalanine ammonia-lyase from *Arabidopsis thaliana*; AtC4H, cinnamic acid hydroxylase from *A. thaliana*; At4CL2, *p*-coumaroyl-CoA ligase from *A. thaliana*; VvVST1, resveratrol synthase from *Vitis vinifera*.

In this study, we focused on developing a resveratrol-producing recombinant industrial *S. cerevisiae*, able to metabolise all sugars present in wine wastes, including the non-naturally metabolised xylose. Then, we aimed at the application of this recombinant strain for the valorisation of vineyard residues, such as hemicellulosic hydrolysate from vine pruning, grape must and wine lees. To accomplish that, we introduced a resveratrol biosynthetic pathway into a xylose-consuming strain, further elucidating the role of PPP in resveratrol production and fine-tuning the nutritional supplementation of the fermentation media for improved resveratrol titres. We also evaluated the impact of simultaneous fermentation of xylose and glucose. This is the first report on the use of renewable carbon sources for resveratrol production from xylose and the use of winery by-products as a substrate to produce this stilbenoid. The expanded multi-sugar utilisation capacity of this yeast is valuable in a biorefinery context, and the obtention of high-value products such as resveratrol is key to increasing the process feasibility following a circular economy concept.

4.2. Materials and methods

4.2.1. Plasmid construction

Plasmid construction in this study was accomplished by USER cloning, according to Jensen et al.[38] A list of plasmids, biobricks and primers used in this study is provided in Tables S1, S2 and S3, respectively. Integrative vectors were generated using the EasyClone-MarkerFree vector set[39] to assemble the different biobricks, and the resultant plasmids were sequenced to confirm proper assembly. *E. coli* strains DH5α competent cells (NZYtech) were used for gene cloning tasks, and the *E. coli* transformants were selected and kept on Lysogeny Broth plates with 100 mg/L of ampicillin.

4.2.2. Yeast strains

All strains used in this study are derivatives from Ethanol Red®, an *S. cerevisiae* commercial strain developed by Fermentis, S.I. Lesaffre for bioethanol fermentation. Yeast transformations were performed according to the lithium acetate protocol.[40] The parent strains were initially transformed with a Cas9-expressing plasmid,[41] followed by a subsequent transformation with the desired recombinant DNA repair fragment and the guide RNA (gRNA) plasmid targeting the required insertion site.[39] The strain L323 is described in Costa et al.[24], expressing the resveratrol biosynthetic pathway (RBP), consisting of the genes *AtPAL2, At4CL2, AtC4H* and *VvVST1*. The strain L326 was developed by insertion of the RBP into a previously engineered xylose-consuming strain developed by Stovicek et al.[35] The strain L543 was

developed by (over)expression of the genes *AtATR2* and *ScCYB5* in the strain L326. A detailed description of the genotype of all the strains is provided in Table S4.

4.2.3. Media and cultivations

The recombinant yeast strains were kept on YPD plates (2% glucose, 2% peptone, 1% yeast extract, 1.5% technical agar). For all the experiments, cell pre-cultures were incubated overnight in 250 mL baffled shake flasks with a working volume of 50 mL of YPD20 (2% dextrose, 2% peptone, 1% yeast extract), with orbital shaking (300 rpm), at 30 °C. Yeast grown overnight was collected at 4000 rpm for 5 min, subsequently washed with sterile deionised water and resuspended in 0.9% sodium chloride, for a final cell concentration of 300 g/L in fresh weight (FW). The initial inoculum was 8 g/L of cells (FW) for all fermentation assays, with biological duplicates. Percentages are weight per volume (w/v).

4.2.3.1. Synthetic media fermentation

Fermentations in synthetic media were carried out in 250 mL baffled shake flasks with a working volume of 50 mL, with orbital shaking (300 rpm), at 30 °C. Concentrated sugar solutions of glucose and xylose (200 g/L) were filter-sterilised and added to the media accordingly to the desired concentrations for each experiment, while the rest of the media components (water, yeast extract and peptone) were autoclaved separately at 121 °C for 15 min in the indicated concentrations. Standard YP supplementation was 10 g/L of yeast extract and 20 g/L of peptone and optimisation of nutritional supplementation ranged from 0 to 50 g/L of yeast extract with no peptone added.

4.2.3.2. Wine wastes fermentations

4.2.3.2.1. Hemicellulosic hydrolysate from vine pruning residue

Vine pruning residue (VPR), collected in May 2019, provided by the Department of Agriculture Research (ITACyL, Finca Zamadueñas, Valladolid, Spain), was used as raw material to obtain a hydrolysate enriched in xylose and glucose. The hemicellulosic hydrolysate of vine pruning residue (VPR) was obtained by hydrothermal treatment, followed by enzymatic hydrolysis of oligosaccharides to obtain fermentable sugars. Hydrothermal treatment was carried out using a liquid to solid ratio (LSR) of 8 kg of water/kg of dried vine pruning residue in a pressurised Parr reactor of 2 L volume in a non-isothermal regime at T_{max} of 215 ºC (corresponding to a severity of 3.89), according to previous optimisation treatment by Jesus et al.[18] The hardness of hydrothermal treatments can be expressed in terms of ''severity" (S_0), defined as the logarithm of the severity factor R_0.[42] After hydrothermal treatment, liquid and solid phases

were separated by filtration to determine solid yield and analysed as described by Jesus et al.[18] The liquid phase (hemicellulosic hydrolysate) was subjected to enzymatic hydrolysis with commercial Cellic CTec2 (kindly supplied by Novozymes Bagsvaerd, Demark), with a xylanase activity of 626 U/mL, for 24 h in an orbital incubator at 45 ºC. Xylanase activity was measured as described by Cunha et al.[43] The hydrolysate was detoxified with activated charcoal at a ratio of 10 g of hydrolysate per 1 g of activated charcoal for 1 h with agitation at room temperature. This step was attained to enable resveratrol on HPLC, due to the considerable phenolic content of hemicellulosic hydrolysates. The activated charcoal was removed by filtration and the hydrolysate was subsequently filter-sterilised. The detoxified VPR hemicellulosic hydrolysate was used as fermentation media containing 14.0 ± 0.3 g/L of glucose, 15.5 ± 0.2 g/L of xylose and 6.1 ± 0.1 g/L of acetic acid, which was supplemented with 7.5 g/L of yeast extract.

4.2.3.2.2. Grape must

Grape must (GM) used in this study are derived from white grapes (variety Verdejo), collected in September 2020, and were provided by the Oenological Station of Castile and Leon - ITACyL (Rueda, Spain) and stored at -20 ºC until used. GM density is 1.09 kg/L and contained composed of 111 g/L glucose, 116 g/L fructose 0.69 g/L total Kjeldahl nitrogen and 0.15 g/L total phenolic compounds, previously characterised by Hijosa-Valsero et al.[14] Grape must pH was adjusted to 6 prior sterilisation, then autoclaved at 121 °C for 15 min. Grape must was dissolved in the fermentation media in percentages from 12.5% to 90% (v/v) and supplemented with 7.5 g/L of yeast extract.

4.2.3.2.3. Wine lees

Wine lees (WL) are derived from red wine, were collected between September and November 2020, and were provided by the Oenological Station of Castile and Leon - ITACyL (Rueda, Spain). WL and have a density of 1.05 kg/L, containing 99.3 g/L ethanol, 12.2 g/L total Kjeldahl nitrogen and 1.51 g/L phenolic compounds (characterised by Hijosa-Valsero *et al*).[14] Wine lees were sterilised by pasteurisation for 1 hour at 60 °C to avoid loss in ethanol content and subsequently sonicated for 30 min to disrupt yeast cells, releasing cytoplasmic contents that act as nitrogen sources for nutritional supplementation of the media, not requiring the addition of commercial yeast extract. Wine lees were dissolved in the fermentation media in percentages from 40% to 80% (v/v).

4.2.4. Analytical methods

Fermentations were monitored by collecting 500 μL of the sample for analysis of sugars, acetic acid, and ethanol, and another 500 μL of the sample was mixed with an equal volume of ethanol (>99% purity), vortexed for 10 s, for the quantification of resveratrol and *p*-coumaric acid. Samples were centrifuged at 13,000 rpm for 5 min, and the supernatants were stored until analysed by HPLC. For sugars, acetic acid, and ethanol, a BioRad Aminex HPX-87H column, at 60 °C, using 5 mM H_2SO_4 as mobile phase and a flow rate of 0.6 mL/min, was used. Peak detection was accomplished using a Knauer-IR intelligent refractive index detector. Resveratrol and *p*-coumaric acid were quantified using a Discovery® HS F5 150 mm × 2.1 mm column (particle size 3 mm), with a flow rate was 0.7 mL/min. A linear gradient was set from 5% to 60% of acetonitrile over 10 mM ammonium formate (pH adjusted by formic acid to 3.0) from 0.5–9.5 min. Resveratrol and *p*-coumaric acid detection were made by absorbance at 333 nm, with retention times of 7.1 min and 6.0 min, respectively, using a diode-array detector. Biomass dry weight (BDW) quantification was accomplished by collecting 1 mL of fermentation broth in previously dried and weighed tubes. The pellet was washed out twice in ethanol and deionised water, sequentially, to remove precipitated resveratrol and residual fermentation media, and incubated at 105 °C for 24 h before being weighed again.

4.2.5. Determination of fermentation parameters

Resveratrol yield on carbon source ($Y_{R/S}$) was calculated as the ratio between the resveratrol concentration at the end of fermentation and the total amount of carbon source consumed over the fermentation. Resveratrol yield on biomass in dry weight ($Y_{R/BDW}$) was determined as the ratio between resveratrol and biomass dry weight concentrations at the end of fermentation. Xylose consumption rates were calculated as the ratio between xylose consumed in a defined period and the duration of that specific period.

4.2.6. Statistical analysis

Statistical analyses were performed using GraphPad Prism. Statistically significant differences between fermentation parameters were tested by repeated measures one-way ANOVA, followed by Tukey post hoc test. Statistically significant differences were established at *p*-value < 0.05. Significance levels: “ns” (non-significant) - $p > 0.05$; * - $p < 0.05$; ** - $p < 0.01$; *** - $p < 0.001$; **** - $p < 0.0001$.

4.3. Results and discussion

4.3.1. Improvement of resveratrol titre by metabolic engineering

The Pentose Phosphate Pathway plays an important role in both xylose metabolism and resveratrol biosynthesis. We started by introducing the heterologous resveratrol biosynthetic pathway (RBP) in a xylose-consuming recombinant Ethanol Red strain, generating the strain L326. In this strain, the 4 genes involved in the non-oxidative PPP reactions (*TAL1, RPE1, RKE1* and *TKL1*) were previously overexpressed.[35] When compared to L323, which is derived from the same Ethanol Red parent strain with the sole expression of the RBP, L326 produced 107.3 mg/L of resveratrol from 20 g/L of glucose, 22% higher than the 88.0 mg/L titre obtained with L323 (Figure **4.2a**). Previous studies have shown that directing the carbon flux to the formation of erythrose 4-phosphate (E4P) through the PPP increases the precursor supply in the shikimate pathway and the aromatic amino acid supply, which are key to resveratrol formation.[44,45] In particular, overexpression of *RKI1* has previously been shown to guide carbon flow toward E4P production while avoiding its recirculation back into glycolysis, and its combination with *TKL1* overexpression further increased shikimate titres.[45]

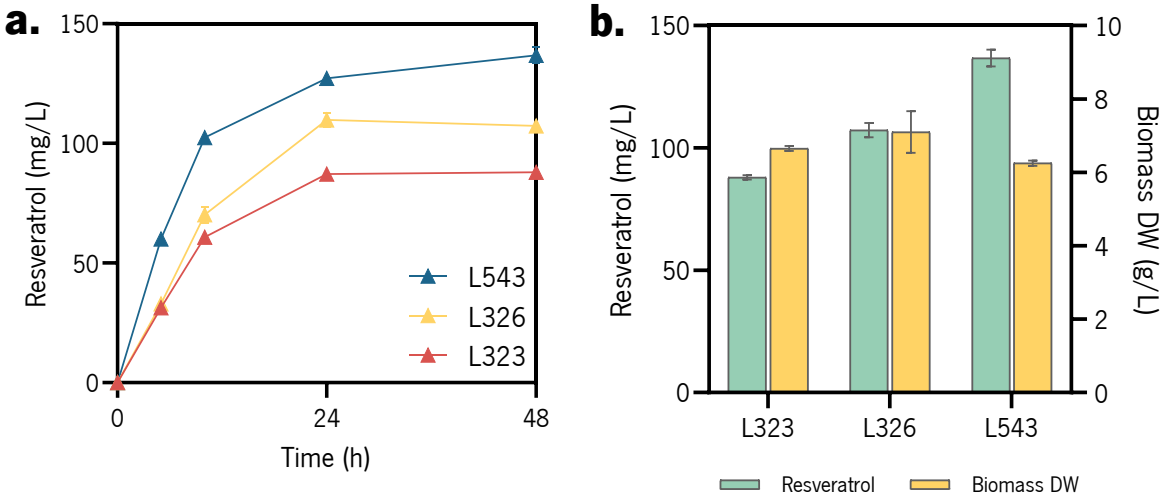

Figure 4.2. Comparison between the resveratrol-producing strains L323 (Ethanol Red with the Resveratrol Biosynthetic Pathway - RBP), L326 (xylose-consuming Ethanol Red with the RPB) and L543 (L326 with overexpression of *CYB5/ATR2*). **a)** Resveratrol production; **b)** Biomass dry weight and maximum resveratrol production at the end of fermentation. Statistical analysis of data points at the end of fermentation: Resveratrol – L323 *vs.* L326 ***; L323 *vs.* L543 ****; L326 *vs.* L543 ****; Biomass DW – no statistically significant (*p*-value > 0.05) between the three strains.

Several cytochrome P450 monooxygenases, such as cinnamate-4-hydroxylase (C4H), a central enzyme in the resveratrol pathway for the conversion of cinnamic acid into *p*-coumaric acid, have known low activity, are dependent on NAD(P)H and require auxiliary proteins for electron transfer.[46] In this sense, we subsequently enhanced the activity of cytochrome P450 by expression of cytochrome B5 (*ScCYB5*) and cytochrome P450 reductase (*AtATR2*) originating the strain L543. This improvement resulted in a resveratrol titre of 136.8 mg/L, an additional increase of 28% compared to the strain L326 (Figure **4.2**). Overexpression of *CYB5* from *S. cerevisiae* and *ATR2* from *A. thaliana* has previously been reported to improve resveratrol production in a laboratory *S. cerevisiae* strain,[8] and our data is in accordance with this previous report. Biomass formation, critical for resveratrol production,[9] does not show statistically significant differences between the 3 strains, hence, resveratrol yield on biomass increased from 13.2 mg/g (L323) to 21.9 mg/g (L543). Therefore, we used the strain L543 in the subsequent fermentations in this study.

4.3.2. Resveratrol production using xylose as sole carbon source

Due to its ability to metabolise xylose, the production of resveratrol exclusively from this pentose sugar was assessed with the previously selected recombinant strain L543. This strain produced 61.3 mg/L of resveratrol from a media with an initial xylose concentration of approx. 20 g/L (YPX20). Nevertheless, the strain could only consume 53% of the xylose present in the fermentation media, with a residual xylose concentration of 9.1 g/L (Figure **4.3**).

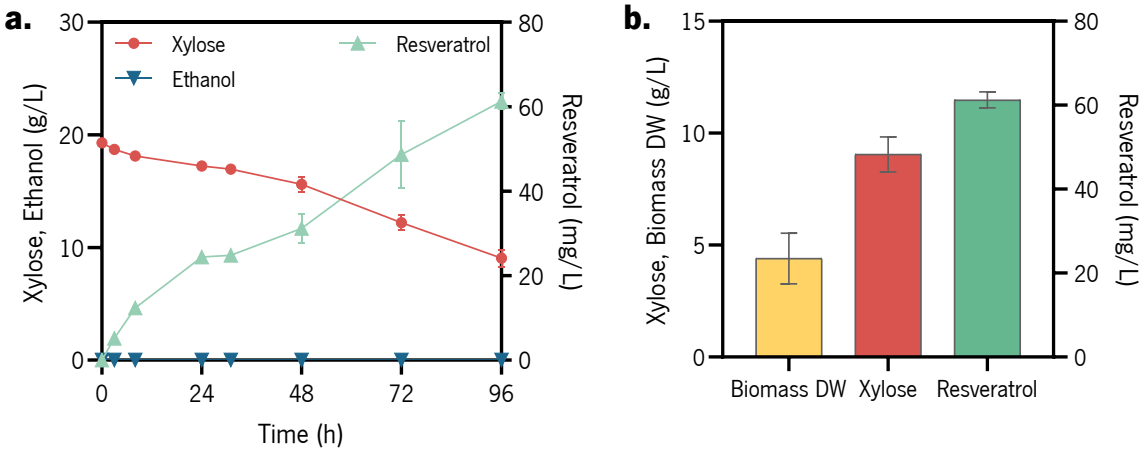

Figure 4.3. a) Fermentation profile of xylose synthetic media by the strain L543; **b)** Biomass dry weight, residual xylose and maximum resveratrol concentrations at the end of fermentation.

The parent strain of L543, a xylose-consuming Ethanol Red strain without the RBP (strain XylC2 V1), was previously shown to have high xylose metabolisation capacity in YPX20 media, being able to consume nearly all xylose in the media in approx. 48 h.[35] The reduced xylose consumption observed in strain L543 might be associated with the metabolic burden caused by the multiple gene integrations in this strain, which has a negative impact on its physiology.[47] Furthermore, high-level expression of the resveratrol biosynthetic pathway genes was previously found to prejudice yeast cell growth.[8,9] Additionally, intracellular accumulation of xylose in yeast cells which are actively metabolising this pentose sugar activates cell mechanisms comparable to carbon limitation, which is often associated with low assimilation rates.[48] Numerous yeast cell defence mechanisms are also triggered in *S. cerevisiae* when in xylose cultivations, such as the upregulation of genes involved in cell starvation, lipid metabolism, stress response and DNA damage.[49] While xylose assimilation capacity does not hinder xylose uptake on the parent strain XylC2 V1, the combination of both RBP and xylose metabolism appears to prejudice xylose consumption by the resveratrol-producing strain L543. No ethanol was observed at any time point probably because xylose consumption was very slow, with an average uptake rate of 0.11 g/L·h for the 96 h of fermentation. As the strain L543 also utilises ethanol to produce resveratrol, it is most likely that the rate at which ethanol is being produced from xylose is lower than the rate of ethanol conversion into resveratrol. Nevertheless, even though the strain was not able to consume all xylose and achieved only 45% of the resveratrol titre on glucose, its yield of carbon consumed was approx. 80% of the yield on glucose (5.6 mg/g of xylose against 6.8 mg/g of glucose).

4.3.3. Evaluation of the effect of nutritional supplementation of the fermentation media on resveratrol production

For increased resveratrol titres, optimisation of the fermentation media supplementation was evaluated. The standard YP supplementation (10 g/L of yeast extract and 20 g/L of peptone) was compared against increasing concentrations of yeast extract (YE) from 0 to 50 g/L (without peptone) in glucose media (Figure **4.4**). Interestingly, the maximum resveratrol concentration of 259.2 mg/L was obtained with supplementation of 7.5 g/L of yeast extract (a 1.87-fold increase compared to YP supplementation), giving a yield of 11.6 mg of resveratrol per g of glucose (Figure **4.4a**). Fine-tuning of the yeast extract supplementation has a high impact on resveratrol production, as the decrease in YE concentration from 7.5 to 5 g/L reduced resveratrol titre by near 4-fold to only 66.8 mg/L. On the other hand, an increase in YE above 7.5 g/L causes a decrease in resveratrol production, with resveratrol titres

declining gradually to 40.6 mg/L when media is supplemented with 50 g/L of YE. Biomass formation is, as expected, correlated to an increase in YE supplementation, as YE is commonly used as supplementation for yeast growth. Even though resveratrol production is highly dependent on biomass, a balance between both appears mandatory for increased yield, as an excessive direction of the carbon to biomass formation penalises metabolite production.

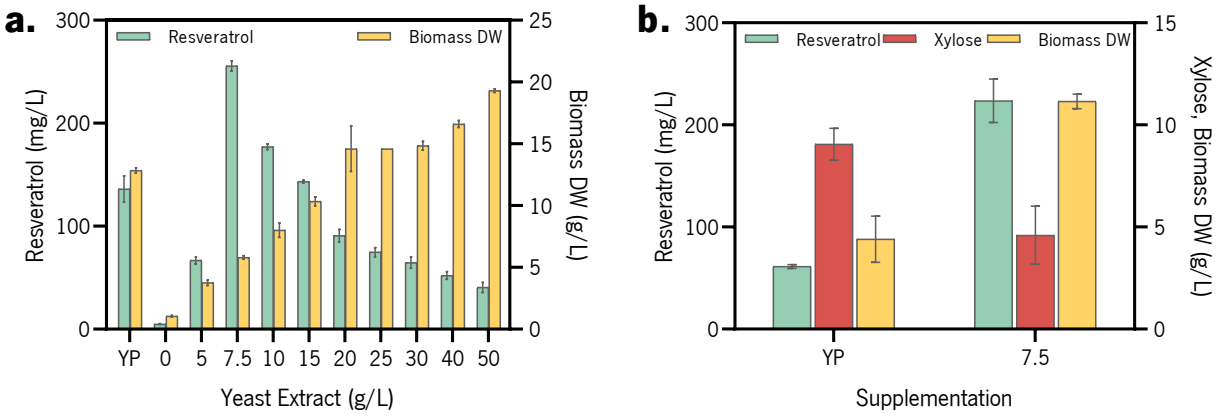

Figure 4.4. Optimisation of media supplementation. **a)** Resveratrol and biomass in dry weight concentrations at the end of fermentation (96 h) in glucose media (20 g/L) supplemented with different concentrations of yeast extract (from 0 to 50 g/L) against the control standard YP supplementation (10 g/L of yeast extract and 20 g/L of peptone); **b)** Resveratrol, xylose and biomass dry weight concentrations at the end of fermentation (96 h) of xylose media (20 g/L) supplemented with 7.5 g/L of yeast extract against the control standard YP supplementation.

We further supplemented a xylose media with these optimal conditions, and while the same trend was observed, the effect of fine-tuning YE supplementation was even more prominent. Supplementation with 7.5 g/L of YE led to a resveratrol titre of 223.6 mg/L, 3.65-fold higher when compared to the control supplementation with 20 g/L of peptone and 10 g/L of yeast extract. This titre is comparable to the one attained in the only previous report on resveratrol production from xylose, which was made using *Scheffersomyces stipitis*, a yeast naturally capable of fermenting this pentose sugar. However, the yield reported in this study is higher. The *S. stipitis* strain produced 248.6 mg/L of resveratrol from 50 g/L of xylose, corresponding to a yield of 4.97 mg of resveratrol per gram of xylose consumed,[50] against 14.5 mg/g achieved using the *S. cerevisiae* L543 (2.92-fold higher).

Interestingly, with the fine-tuning of supplementation, the resveratrol yield on xylose was higher than the resveratrol yield on glucose (14.5 mg/g of xylose consumed against 11.6 mg/g of glucose consumed). Even though resveratrol titres on glucose media were 1.16-fold higher with the supplementation of 7.5 g/L of YE compared to xylose fermentation, the differences were minimised compared to the fermentation with standard YP supplementation (resveratrol titre on YPD20 was 2.23-fold higher than on YPX20). The enhanced xylose consumption observed after the fine-tuning of the supplementation (1.41-fold higher) can help to explain this. Also, contrarily to glucose fermentation, biomass formation with 7.5 g/L of YE supplementation in xylose media was 2.53-fold higher than with YP supplementation. Another possible explanation for the different behaviour between glucose and xylose fermentation may rely on the fact that *S. cerevisiae* is Crabtree-positive, fermenting glucose into ethanol under aerobic conditions. This effect does not occur in xylose fermentation in yeast due to the requirement of high metabolic flux.[51] This means that in high aeration conditions (such as resveratrol production processes), glucose fermentation is more prone to direct the carbon flow to other metabolic routes than xylose fermentation. Sun et al.[52] reported that, for the production of isoprenoids (also derived from acetyl-CoA), the lack of Crabtree-effect in xylose cultivation led to an increase of 53% in biomass formation, with higher yields on carbon when compared to glucose cultivation. Improved biosynthesis of acetyl-CoA-derived products was also attained by exploiting xylose as a carbon source, due to the facilitated supply of acetyl-CoA in the cytosol.[53] Data in Figure **4.4** shows that supplementation of the media with 7.5 g/L of yeast extract is more favourable to resveratrol production than the standard YP supplementation in both glucose and xylose cultivations. The fine-tuning of the media supplementation is attractive for the increase of resveratrol titres causing a reduction in operation costs, which is mandatory in an industrial context. Therefore, we used 7.5 g/L of yeast extract in further experiments of this work where supplementation was necessary.

4.3.4. Co-fermentation of xylose and glucose for increased resveratrol titres

The Pentose Phosphate Pathway (PPP) is crucial for both resveratrol production and xylose assimilation (Figure **4.1**). We hypothesised that co-fermentation of xylose and glucose in the media might benefit the resveratrol production by pushing the metabolic flux through PPP, therefore improving the resveratrol producing pathway. We compared a fermentation medium with 62 g/L (YED) against a medium with 50 g/L of glucose and 10 g/L of xylose (YEDX), for a matched total carbon molarity of 344

mM in both media. Simultaneous fermentation of glucose and xylose led to an increase of 1.31-fold in resveratrol production when compared to sole glucose fermentation (Figure **4.5**).

In both fermentations, all glucose was consumed in the first 12 h, with an accumulation of ethanol in the fermentation media of around 17 g/L in both conditions. Interestingly, resveratrol concentration at 12 h in YED fermentation was only 69.2 mg/L, while YEDX fermentation produced 186.2 mg/L in the same timeframe, with a total carbon consumption of 53.8 g/L (50.1 g/L of glucose plus 3.7 g/L of xylose). Resveratrol production in glucose fermentation relies mainly on the ethanol phase. The ethanol produced from glucose fermentation is consumed and converted into resveratrol, representing approx. 76% of the overall resveratrol production in this condition. Our observation is in accordance with previous studies on *de novo* resveratrol production from glucose.[7,8] On the other hand, while simultaneously fermenting glucose and xylose, the yeast achieved approx. 48% of the overall production in the first 12 h, increasing the resveratrol yield of the sugar phase. After glucose depletion, both conditions produced similar resveratrol concentrations of approx. 200 mg/L. The increased resveratrol titre associated with YEDX fermentation is probably related to the abovementioned influence of the PPP in the resveratrol pathway. It is estimated that only up to 2.5% of the glucose is metabolised via the oxidative PPP by *S. cerevisiae*, and another 10 to 20% can enter the non-oxidative PPP via the glycolytic metabolites (see Figure **4.1**).[54,55] On the other hand, xylose enters the cell metabolism via xylulose-5-phosphate, part of the non-oxidative PPP, essential in generating E4P for the shikimate pathway and, ultimately, in phenylalanine formation. In this sense, it is most likely that the presence of xylose in the fermentation media pushes the PPP activity in the yeast, increasing resveratrol titres (Table **4.1**).

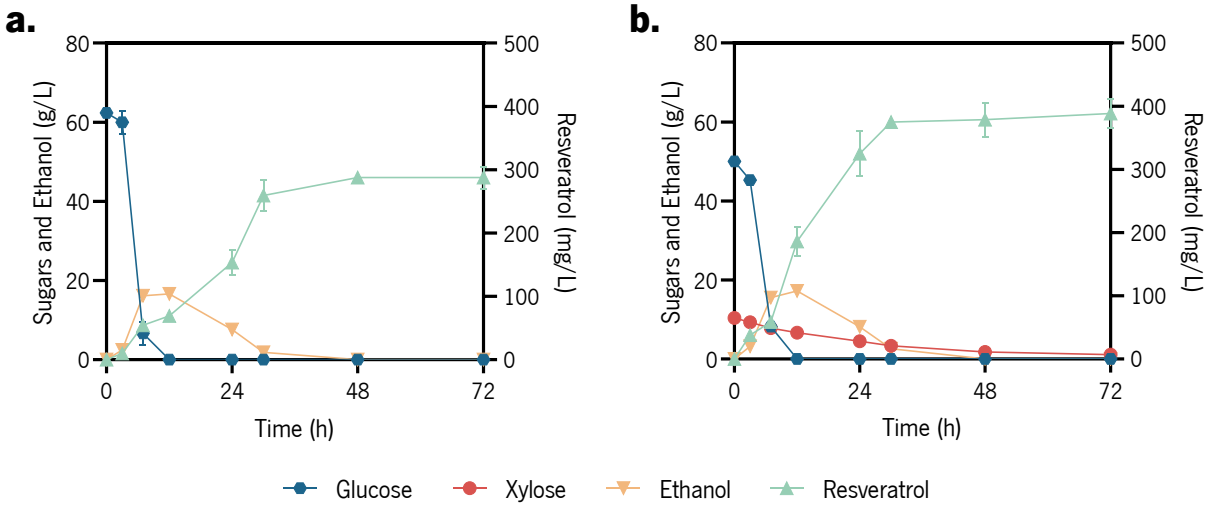

Figure 4.5. Fermentation profiles of YE media with **(a)** 62 g/L of glucose and **(b)** 50 g/L of glucose together with 10 g/L of xylose both fermentations with total carbon molarity of 344 mM.

Even though a higher resveratrol titre was achieved in YEDX fermentation, a residual concentration of 1.1 g/L of xylose was observed at the end of fermentation (89% of the xylose consumed). Xylose uptake in yeast cells is facilitated by hexose transporters, unspecific for pentose sugars,[56] but high concentrations of glucose outcompete xylose, which represses the simultaneous co-consumption of glucose and xylose.[57] Glucose is the preferred source of energy for *S. cerevisiae*, being usually consumed prior to any other carbon source.[58] This results in diauxic growth, with an initial fast assimilation of glucose followed by a slower uptake of other carbon sources.[59] Furthermore, a previous study reported that after all glucose in fermentation media with both glucose and xylose, common in lignocellulosic processes, the xylose consumption rate is considerably reduced to rates lower than the ones observed in xylose-only media.[60] Indeed, we observed that, after glucose depletion, the xylose consumption rate drops from 0.31 g/L·h to 0.09 g/L·h. Only 2.3 g/L of xylose were consumed after 30 h of fermentation, which did not lead to an increase in resveratrol concentration and was probably channelled to cell maintenance. A glucose-limited fed-batch fermentation strategy, used, e.g., to increase xylitol productivity,[26,61] could be an interesting approach to aid in the full consumption of xylose, by avoiding catabolite repression, possibly leading to even higher resveratrol titres.

Table 4.1. Fermentation parameters of glucose and co-fermentation of glucose and xylose in YE media.

Condition	G_0 (mM)	X_0 (mM)	R_{max} (mg/L)	BDW (g/L)	$Y_{R/S}$ (mg/g)	$Y_{R/BDW}$ (mg/g)
D62	344	0	287.5 ± 17.7	10.6 ± 0.0	4.6 ± 0.3	27.1 ± 1.7
D50X10	277	67	388.4 ± 23.9	11.8 ± 0.4	6.5 ± 0.0	31.8 ± 1.3

G_0, initial glucose concentration; X_0, initial xylose concentration; Rmax, resveratrol concentration at the end of fermentation; BDW, biomass concentration in dry weight at the end of fermentation; $Y_{R/S}$, yield of resveratrol on carbon source; $Y_{R/BDW}$, yield of resveratrol on biomass dry weight

4.3.5. Valorisation of wine wastes as carbon sources for resveratrol production

As abovementioned, the wine industry generates multiple wastes on a large scale, which can be used for several biotechnological processes. Among them, it is possible to obtain several different carbon sources. Grape must (GM) has a high content of glucose and fructose (approx. 11% (w/v) of each) and wine lees (WL) are rich in ethanol (approx. 10% (w/v)). Additionally, the hemicellulosic hydrolysate from

hydrothermal treatment of vine pruning residues (VPR) has similar content of glucose (14 g/L) and xylose (15.5 g/L). The strain L543 can use all these carbon sources to produce resveratrol (Figure **4.1**) and, therefore, we applied it to different wine valorisation processes.

4.3.5.1. Resveratrol production from hemicellulosic hydrolysate derived from hydrothermally pretreated vine pruning residue

As shown in data from Figure **4.5**, simultaneous consumption of glucose and xylose favours resveratrol production. In this sense, vine pruning residue (VPR) hydrolysate is a very promising substrate for resveratrol production, as it has a relatively high amount of glucose (14 g/L) when compared to other hydrolysates like eucalyptus wood or corn cob,[24] allied to a xylose concentration of 15 g/L. The acetic acid present in the VPR hydrolysate (approx. 6.1 g/L) can also be used for resveratrol production through acetate metabolism (Figure **4.1**), but a concentration in this range is known to have a negative impact on yeast physiology.[62] This attributes even more importance to the choice of a robust chassis, such as Ethanol Red, that has demonstrated effective usage of lignocellulosic substrates.[43,63–65] In Figure **4.6**, we see that the strain L543 is capable of producing 167.1 mg/L of resveratrol after 96 h of fermentation in detoxified VPR hemicellulosic hydrolysate (93% of fermentation media volume, plus inoculum and media supplementation), accumulating 42.3 mg/L of *p*-coumaric acid at the end of fermentation. All glucose in the media was consumed, as expected, but only 8.4 g/L of xylose was consumed (residual 6.6 g/L). This is aligned with the previous experiments in this study, where this strain only seemed to be capable of consuming up to 10 g/L of xylose in the media in 96 h. A concentration of 1.97 g/L of acetic acid was observed at the end of fermentation, meaning that the strain partially utilised the acetic acid present in the hydrolysate. No ethanol was observed at the end of fermentation, which again may be related to the accumulation of *p*-coumaric acid after ethanol depletion. Here we demonstrate the feasibility of a hemicellulose-to-resveratrol process, and possible combinations between the different wine residues might further contribute to increased resveratrol yields.

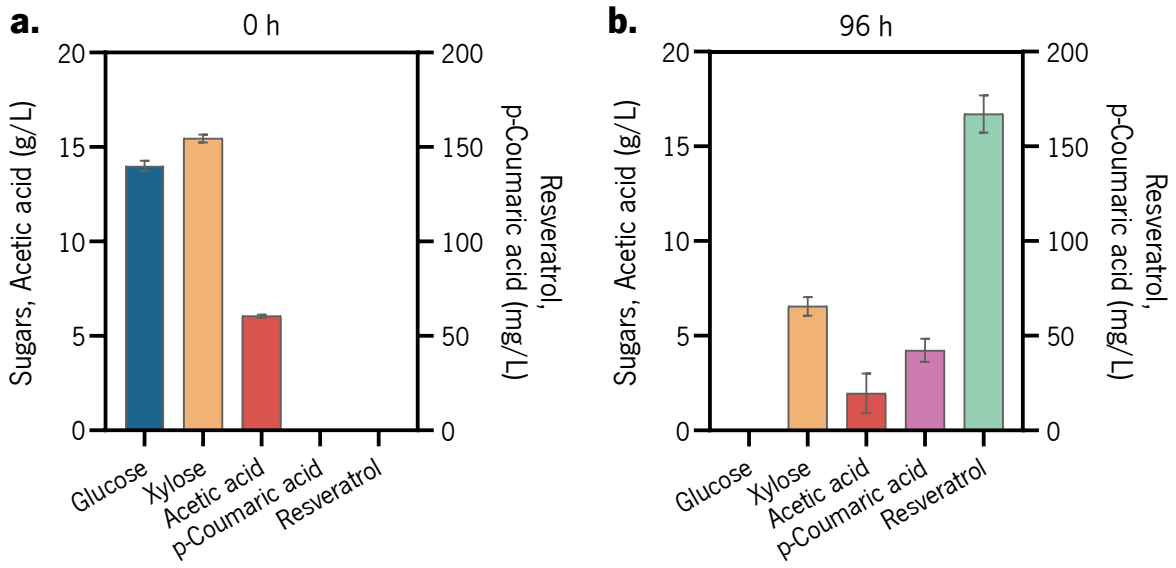

Figure 4.6. Vine pruning residue fermentation data points of **(a)** sugars and acetic acid at the beginning of the fermentation and **(b)** sugars, acetic acid, *p*-coumaric acid and resveratrol at the end of fermentation.

4.3.5.2. Resveratrol production from glucose and fructose mixture derived from grape must

Initially, the maximum volume possible of grape must dissolved in the fermentation media was attempted, using 95% of grape must (GM) in the fermentation media (103.7 g/L of glucose and 111.5 g/L of fructose), with the remaining volume accounted for inoculum and media supplementation. A resveratrol production of only approx. 50 mg/L was attained, which is relatively low when considering a total initial carbon availability of more than 200 g/L of both glucose and fructose. This was mainly due to the accumulation of high levels of glycerol and acetic acid. After 24 h almost 10 g/L of acetic acid were found in the media, which practically ceased ethanol consumption and *p*-coumaric acid conversion (Figure A4.1). Grape must is very acidic, and even though the initial pH was adjusted to 6 and calcium carbonate was added to neutralise the media, the final pH was still very low (approx. 3). Given this, we reduced the GM content in the media to half of the fermentation media to have milder conditions, and this led to an over 4-fold increase in resveratrol titre, to 212.3 mg/L, using 50% of GM in the fermentation media (54.7 g/L of glucose and 58.9 g/L of fructose). In this case, no accumulation of by-products, ethanol or *p*-coumaric acid was observed at the end of fermentation, with a final pH of 4.22 (Figure **4.7a**).

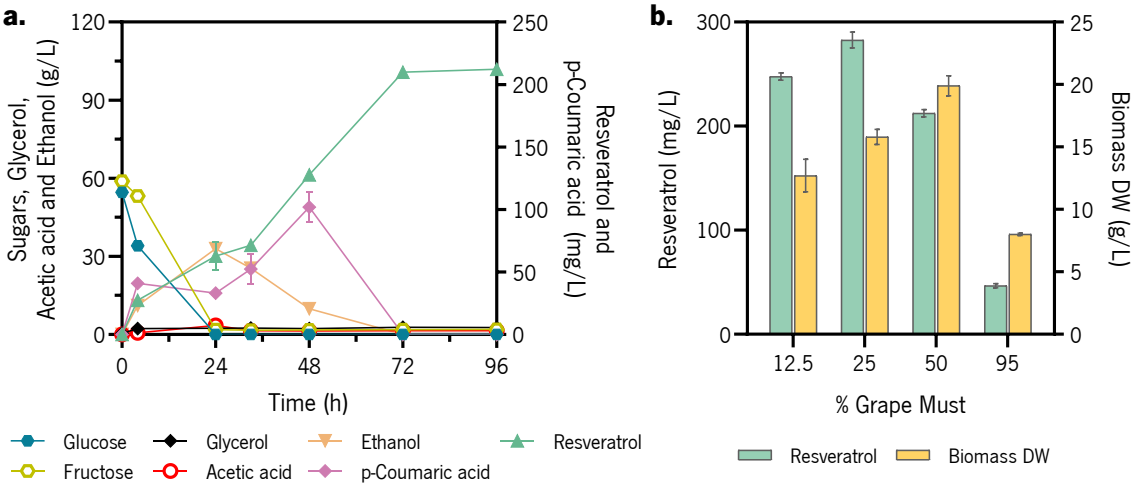

Figure 4.7. Fermentation of grape must. **a)** Fermentation profile of media with 50% grape must (v/v); **b)** Resveratrol concentration at the end of fermentation (96 h) using a range of grape must dissolved in the media from 12.5 to 95%.

Further on, we attempted to use other percentages of GM in the media. A maximum resveratrol titre of 282.7 mg/L was obtained using 25% of GM after 96 h of fermentation (27.5 g/L of glucose and 29.3 g/L of fructose), corresponding to an initial carbon source availability of nearly 60 g/L total (Figure **4.7b**). Nevertheless, the highest resveratrol yield of 8.8 mg/g of sugar was obtained using 12.5% of GM (13.7 g/L of glucose and 14.4 g/L of fructose), against the yields of 5.0 mg/g using 25% and 1.9 mg/g using 50%. This follows the data obtained in synthetic media where, despite an increase in resveratrol titres by increasing glucose concentration from 20 (section 3.3) to 62 g/L (section 3.4), resveratrol yield on glucose decreased from 11.6 mg/g to 4.6 mg/g. The increased yields associated with a lower percentage of GM dissolved in the media are also valuable from the point-of-view of cost reduction and optimisation of process conditions. Using lower concentrations of GM also eliminated the need to add calcium carbonate ($CaCO_3$) to neutralise the fermentation media. The final pH in the fermentation with 12.5 to 50% of GM was between 4.36 and 4.22, respectively. This also helps in preserving resveratrol stability, as resveratrol is more stable under acidic conditions, up to pH 6.[66] On the other hand, fermentation of 95% of GM without $CaCO_3$ is not possible, as the pH drops very sharply, and the strain is not able to produce resveratrol (data not shown). Even with the addition of 5 g/L of $CaCO_3$, the final pH for the fermentation of 95% was 4.97. Interestingly, with a lower percentage of GM in the media (up to 25%), when $CaCO_3$ was added to the fermentation media, resveratrol production slightly decreased (Figure A4.2).

4.3.5.3. Resveratrol production exclusively from ethanol derived from wine lees

Wine lees (WL) are an interesting substrate for resveratrol microbial production due to their high content of ethanol (around 100 g/L). Ethanol can act as a carbon source for resveratrol production, increasing the pool of malonyl-CoA, a direct precursor of resveratrol (see Figure 4.1), and its use as a carbon source, always coupled with glucose, has been already demonstrated in *S. cerevisiae*.[6–8] Here, we show the feasibility of using WL as a cheap substrate to produce resveratrol exclusively from ethanol, which has not been demonstrated before. A maximum resveratrol titre of 263.9 mg/L was attained with 50% of WL in the fermentation media (49.4 g/L of ethanol). Above 70% of WL (68.4 g/L of ethanol) dissolved in the fermentation media no resveratrol production was observed (Figure **4.8a**). Dissolution of 80% of WL resulted in excessive viscosity of the fermentation medium, which created constrains in mass transfer. Furthermore, the initial ethanol concentration available in this condition was 78.5 g/L, which was previously found to be inhibitory for cell growth when using the strain L323, the background strain of L543.[7] Given this, no resveratrol production was attained above 80% of WL dissolved in the media.

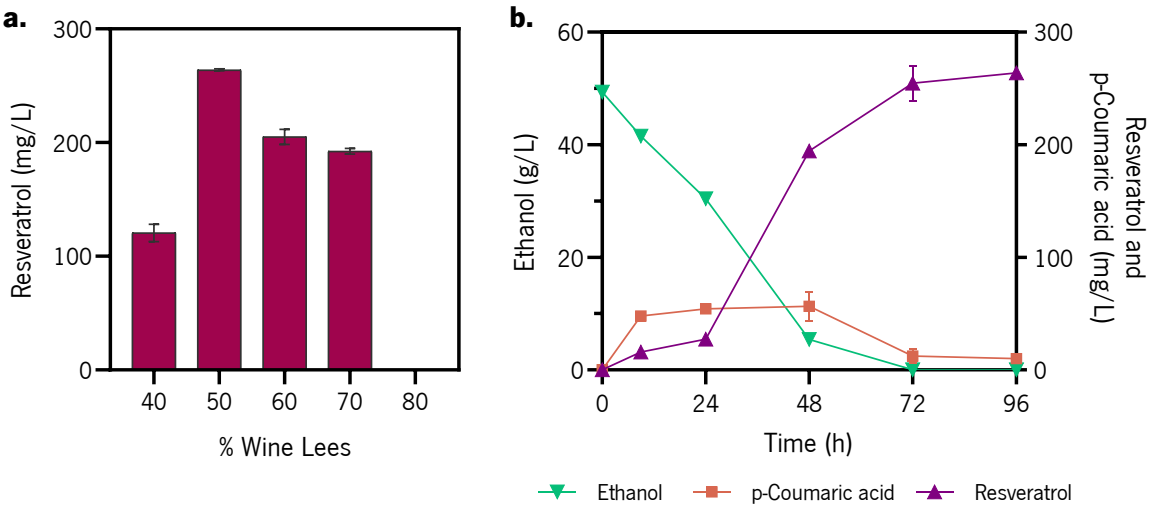

Figure 4.8. Fermentation of wine lees using ethanol as the sole carbon source. **a)** Resveratrol concentration after 96 h in media with a percentage of dissolved wine lees from 40 to 80% (v/v); **b)** Fermentation profile of media with 50% (v/v) of wine lees dissolved.

Data in **Figure 4.8b** shows that all ethanol in the media with 50% of WL is consumed in 72 h. A residual concentration of 10 mg/L of *p*-coumaric acid was observed at the end of fermentation (96 h). It is worth noticing that, after ethanol depletion, *p*-coumaric acid conversion ceased and no additional resveratrol was produced. In a parallel experiment, the addition of 10% of GM to provide a source of glucose and fructose showed no considerable difference in resveratrol production (approx. 270 mg/L)

but rather increased the residual *p*-coumaric acid at the end of fermentation. Again, we observed that after ethanol depletion, the conversion of *p*-coumaric acid into resveratrol stops, which ultimately led to its accumulation (Figure A4.3). One possible explanation for this might hinge on an imbalance between the precursors needed for resveratrol formation. Resveratrol formation depends directly on the precursors *p*-coumaroyl-CoA and malonyl-CoA. The first one is obtained through phenylalanine-derived *p*-coumaric acid, while malonyl-CoA is derived from acetate, which by its turn can be obtained through ethanol metabolism (see Figure **4.1**). To generate 1 molecule of resveratrol, 1 molecule of *p*-coumaroyl-CoA and 3 molecules of malonyl-CoA are necessary,[9] and the lack of malonyl-CoA supply after ethanol depletion most likely cease *p*-coumaric conversion to resveratrol to the imbalance of both branches. This way, fine-tuning the balance between both branches of the precursor supply of the resveratrol biosynthetic pathway is likely necessary for optimal resveratrol yields. Table **4.2** compiles the main fermentation parameters of the experiments using wine wastes as substrate.

Table 4.2. Fermentation parameters from experiments using wine wastes as substrate.

	Carbon source (g/L)					
Residue	**Glucose**	**Xylose**	**Fructose**	**Ethanol**	**R_{max} (mg/L)**	**$Y_{R/S}$**
Vine pruning residue	13.0 ± 0.3	14.4 ± 0.2	-	-	167.1 ± 9.8	7.3 ± 0.4
Grape must						
12.5%	13.7 ± 0.2	-	14.4 ± 0.6	-	247.6 ± 3.5	8.8 ± 0.0
25%	27.5 ± 0.6	-	29.3 ± 0.9	-	282.7 ± 7.6	5.0 ± 0.3
50%	54.7 ± 1.8	-	58.9 ± 1.8	-	212.3 ± 3.5	1.9 ± 0.0
95%	103.7 ± 2.3	-	111.5 ± 2.7	-	46.5 ± 2.0	0.2 ± 0.0
Wine lees						
40%	-	-	-	40.3 ± 0.4	120.6 ± 7.6	3.0 ± 0.2
50%	-	-	-	49.4 ± 1.1	263.9 ± 0.9	5.3 ± 0.1
60%	-	-	-	60.2 ± 2.6	205.0 ± 6.6	3.4 ± 0.0
70%	-	-	-	68.4 ± 2.7	192.4 ± 2.4	2.8 ± 0.1
80%	-	-	-	78.5 ± 0.8	0.0 ± 0.0	0.0 ± 0.0

R_{max}, resveratrol concentration at the end of fermentation; $Y_{R/S}$, yield of resveratrol on carbon source

4.3.6. Overall balance of wine wastes processing for resveratrol production

According to the Food and Agriculture Organization of the United Nations (FAO) and the OIV, on average, approximately 75 million tonnes of grapes are produced every year. Roughly, for each hectare of vineyard surface area, it is possible to obtain 10 tonnes of grapes.[67] The mandatory pruning of vine trees generates around 5 tonnes per hectare of vineyard per year.[68] From the total grape production, approximately 4.79 tonnes of grapes per hectare of surface area are pressed for wine production, while 493 kg of pressed grapes per hectare are not used for wine production, but rather used for grape must and juice production.[67] On average, 1.3 kg of grapes are necessary to produce 1 L of wine, and 6% of the total grape content is wasted in the form of wine lees,[68] resulting in the accumulation of around 287 kg of wine lees per hectare of vineyard, which can contain approx. 27 kg of ethanol (depending on the type of wine).

Therefore, taking into account the results obtained for resveratrol production from the abovementioned wine wastes (Table **4.2**) and considering the amount of vine pruning residue, grape must and wine lees that are generated, Figure **4.9** shows the kg of resveratrol that can be produced per hectare of vineyard. As seen, approx. 7 kg of resveratrol could potentially be produced from the vine pruning residues generated by each hectare cultivated. On the other hand, grape must (not used for wine production) and wine lees could yield 1.11 kg and 0.148 kg per ha, respectively. Nevertheless, the highest yield (measured as g of resveratrol per kg of biomass) was obtained for grape must (2.259 g resveratrol/kg grape must), followed by 1.404 g and 0.515 g of resveratrol per kg of vine pruning residue and wine lees, respectively. These results obtained through a biotechnological process are further emphasised when compared with resveratrol obtained via plant extraction from other wine residues, such as the skin from red grapes, one of the largest sources of resveratrol in nature, with extraction yields reported ranging between 5-10·10^{-2} g of resveratrol/kg of grape skin.[69]

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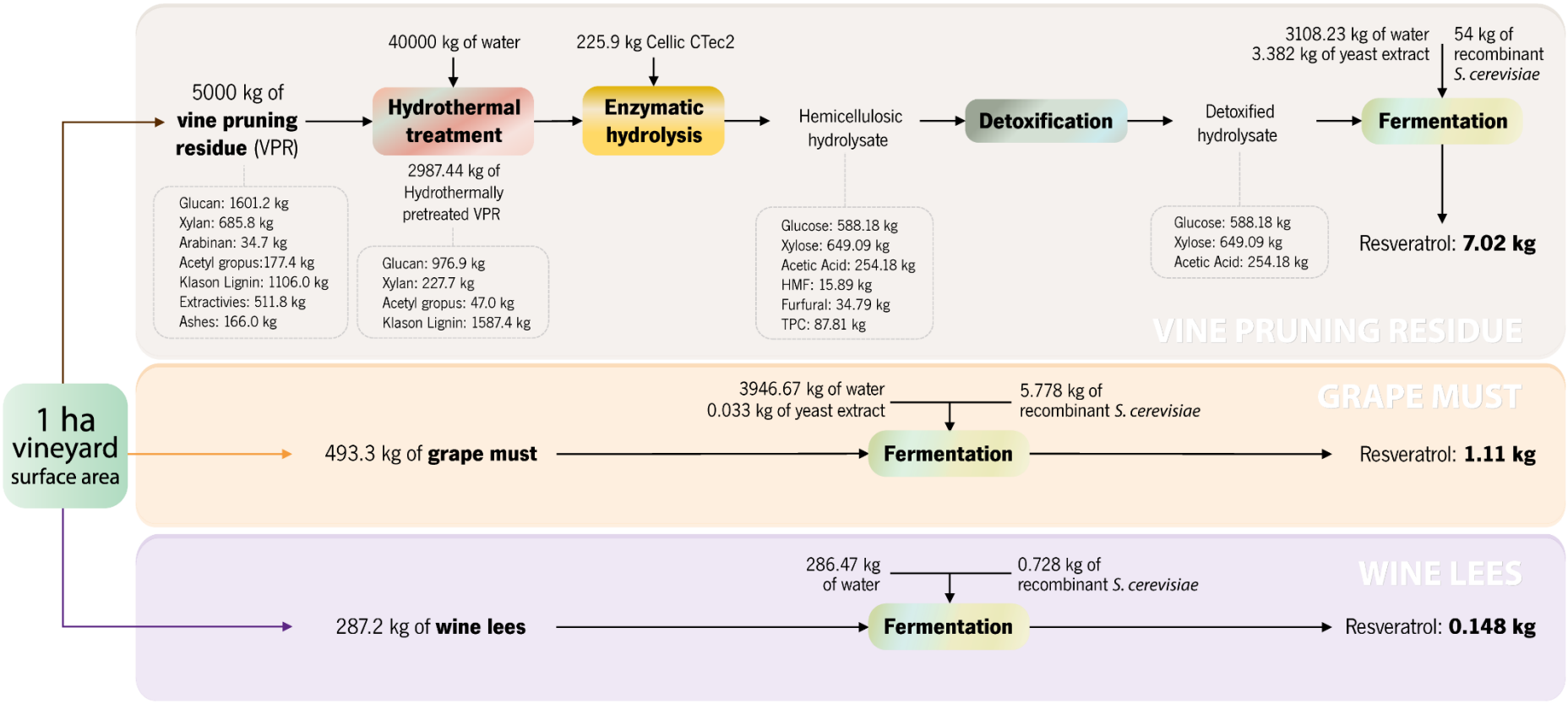

Figure 4.9. Overall balance of resveratrol biosynthesis by recombinant *S. cerevisiae* from vine pruning residues, grape must and wine lees produced by each hectare of vineyard cultivated.

4.4. Conclusions

In this work, an *S. cerevisiae* strain was, for the first time, utilised as a host for resveratrol production from xylose, the second most abundant sugar in nature, which enabled the usage of a wide range of substrates. The significant role of the non-oxidative part of the pentose phosphate pathway in the resveratrol biosynthesis was shown. Valuable insights on the effect of coupling xylose with glucose fermentation are provided, where the benefits of simultaneous fermentation of xylose and glucose over glucose-only media are highlighted by a 1.31-fold increase in resveratrol production, reaching a titre of 388 mg/L from a mixture of both carbon sources. Here, following the circular bioeconomy concept, the palette of possible substrates for resveratrol production was expanded by repurposing several residues from the wine industry. Resveratrol production exclusively using ethanol obtained from wine lees, as well as the simultaneous use of glucose and fructose from grape must, were achieved, attaining competitive titres above 250 mg/L. Additionally, this is also the first report on the development of a hemicellulose-to-resveratrol process, by fermentation of a detoxified vine pruning residue hydrolysate. At its core, the concept of biorefinery comprehends the conversion of renewable materials into value-added compounds, allowing for full resource usage. This work contributes significantly to further advances toward the integration of industrial residues for high-value compound production promoting a greener future in bioprocess development.

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Chapter V.

Conclusions and Future Perspectives

The environmental problem of the extensive fossil resources consumption is, more than ever, accelerating the shift towards the production of biobased compounds from renewable sources. The biorefinery concept is, at its core, deeply connected to biofuels production, such as bioethanol, but expanding its possible outcomes by exploring novel processes and products to be integrated into the circular economy principle is mandatory not only for a greener future but for a sustainable (and attainable) one. Due to its remarkable potential as a nutritional supplement and therapeutic agent, resveratrol has been gaining interest as a target chemical for several industries. Nevertheless, so far, the focus of research studies has been mainly driven towards the use of synthetic media and mainly using laboratory yeast strains. While this is necessary to unravel novel pathways and optimise the overall yield of resveratrol microbial biosynthesis, the incorporation of high-value low-volume chemicals is key to the successful establishment of a biorefinery, addressing its economic goal. The main intent of this thesis was to contribute to the valorisation of agro-industrial wastes, repurposing them to produce compounds of interest, namely resveratrol. Given this, the main outcomes of this study are summarised below.

The feasibility of a cellulose-based resveratrol bioprocess was demonstrated. To attain this, a set of selected industrial *Saccharomyces cerevisiae* strains was successfully engineered, via the CRISPR/Cas9 system, and assessed for resveratrol production at high temperatures. This allowed the identification of potentially good candidates for their application to lignocellulosic processes. By combining the heterologous resveratrol biosynthetic pathway with a robust industrial background, simultaneous saccharification and fermentation of hydrothermally pretreated eucalyptus wood was successfully carried out at 39 ºC for resveratrol production, which considerably values the process.

Microbial production of resveratrol using lactose as a carbon source was here reported for the first time in the literature. By carefully addressing the balance of the expression of a heterologous lactose permease and β-galactosidase from *Kluyveromyces lactis*, under the control of promoters with distinct relative strength, together with the expression of the resveratrol biosynthetic pathway in an appropriate yeast chassis, known for its aptitude to co-consume glucose and galactose, the recombinant strain exhibited excellent lactose consumption even at very high lactose concentrations. This enabled the utilisation of cheese whey, a residue from the dairy industry, as a substrate.

Also, resveratrol production from xylose using *S. cerevisiae* was newly reported, enabling the utilisation of this abundant pentose sugar allied to the notable fermentative capacity of this host. As xylose is necessarily metabolised through the pentose phosphate pathway (PPP), which was found to have also a positive impact on resveratrol production, simultaneous utilisation of glucose and xylose was

demonstrated to lead to higher titres than glucose alone, further highlighting the potential of this strain for application in hemicellulosic processes.

The expanded sugar utilisation of the recombinant xylose-consuming resveratrol-producing *S. cerevisiae* strain allowed the valorisation of important agro-industrial wastes from the wine industry, such as vine pruning residue hemicellulosic hydrolysate (glucose and xylose), grape must surplus (glucose and fructose) and wine lees (ethanol).

Throughout this study, several strategies were applied to enhance the resveratrol titres and yields. This encompassed various key topics for the resveratrol fermentation process, such as enhancement of the precursor supply of the resveratrol pathway in the yeast cell, fine-tuning the oxygenation conditions through a two-stage process and optimising the nutritional supplementation of the media, further increasing the economic viability of the overall process, either by increasing the yield of the process or reducing its costs.

When taken all into account, the integration of agro-industrial wastes for resveratrol bioprocesses has demonstrated potential for future implementation. The development of yeast strains with expanded sugar utilisation ability unfolds novel applications in this field, as it enables the development of process configurations that would not be feasible only with naturally metabolised sugars, e.g., whole slurry fermentation with simultaneous utilisation of cellulose (glucose) and hemicellulose (xylose)[1] or multi-waste valorisation processes like cheese whey (lactose) supplementation to LCM fermentation (glucose and/or xylose).[2] Combining the multiple outcomes of this thesis could generate even more value for this proposal. There is still a lot of room for improvement, but the vast quantities of waste generated could already be used for producing large quantities of resveratrol. For instance, the strain constructed in Chapter II would allow the production of 2.12 kg of resveratrol per tonne of Eucalyptus wood. By its turn, per each tonne of the nearly 200 million tonnes of cheese whey yearly wasted by the dairy industry, it would be possible to produce approx. 0.14 kg of resveratrol using the recombinant strain reported in Chapter III. Finally, per each hectare of cultivated area for wine production would be possible to produce 7.54 kg of resveratrol from vine pruning residues, 1.11 kg from grape must surplus and 0.148 kg from wine lees using the xylose-consuming resveratrol-producing strain reported in Chapter IV. Figure **5.1** compiles all the carbon sources and respective agro-industrial residues used in this study.

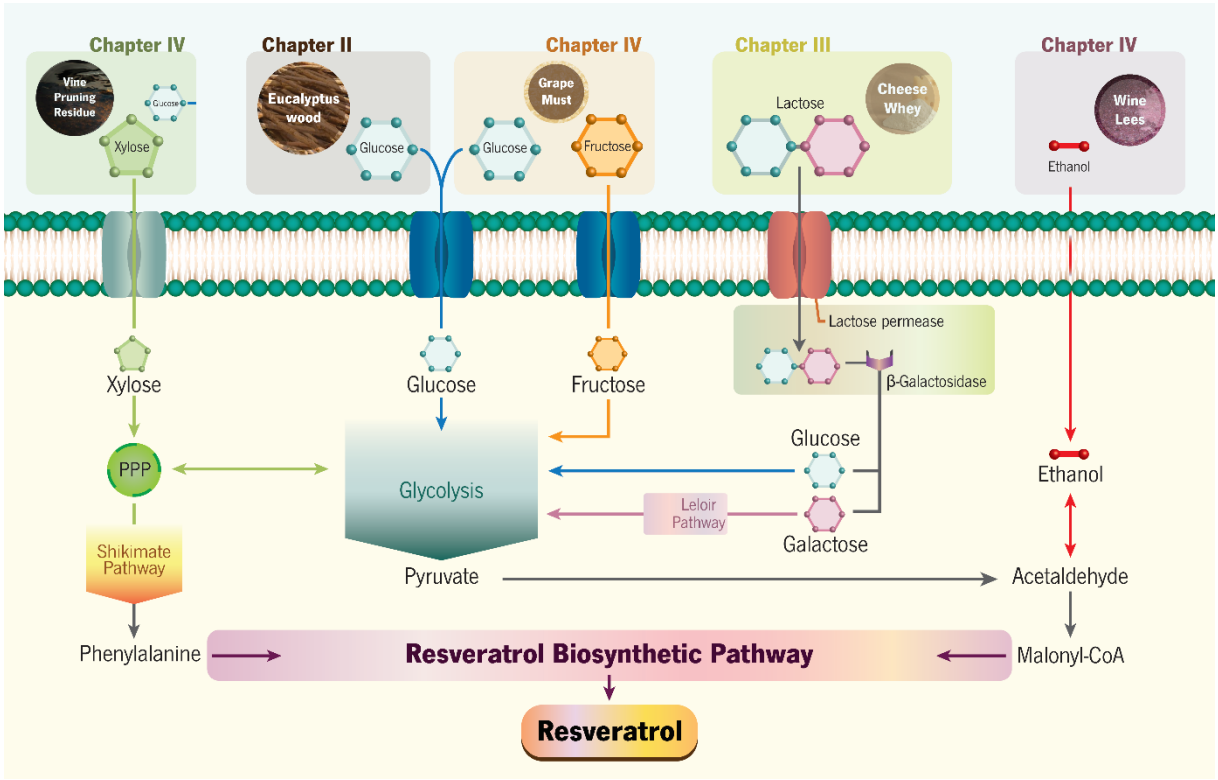

Figure 5.1. Summary of all the agro-industrial wastes used in this study. Chapter II, eucalyptus wood; Chapter III, cheese whey; Chapter IV, vine pruning residue, grape must surplus, wine lees.

Limited yields and productivities associated with conventional fermentation processes and the economy and sustainability of the materials used are identified among the major challenges in fermentation engineering.[3] For the integration of resveratrol processes in the biorefinery context, the immediate priority for future research should rely primarily on two main areas: selective (more than extensive) engineering of the resveratrol biosynthetic pathway and its precursor supply; and optimisation of the fermentation process for enhanced titres, mandatory for implementation at an industrial scale. These should be addressed hand in hand, rather than from a single point-of-view. A well-developed bioprocess needs a suitable microorganism to maximise its potential, while the opposite is also true.

Future studies should focus on enhancing the metabolic pathway for resveratrol production, which is vital to achieving higher yields. The shikimate pathway is crucial for resveratrol production, starting with the condensation of erythrose-4-phosphate, one of the intermediates of the non-oxidative PPP, and phosphoenolpyruvate, derived from glycolysis, into phenylpyruvate. Given this, implementing strategies to increase the levels of both precursors, as well as other reactions in the shikimate pathway, should be explored in future studies for increased resveratrol titres. Tuning the pyruvate kinase activity by expressing alternative variants,[4] the use of the mutated feedback-resistant versions of *ARO3*^{K222L}, *ARO4*^{K229L} and

ARO7^{G141S}, involved in the shikimate pathway intermediate reactions,[4–6] as well as the elimination of competing pathways, e.g., by deleting *ARO10*, codifying for phenylpyruvate decarboxylase,[5,6] has also been shown to have a positive impact on aromatic amino acids formation or resveratrol production. Overexpression of *ZWF1,* coding for a cytosolic glucose-6-phosphate dehydrogenase involved in the oxidative PPP (allied to the expression of NADH kinase encoded by *POS5*), was previously shown to improve the biosynthesis of carotenoids by intensifying the regeneration of the key NADPH co-factor.[7] As cinnamate 4-hydroxylase (C4H) is dependent on NADPH, being a bottleneck of the conversion of phenylalanine into *p*-coumaric acid in the resveratrol biosynthetic pathway, this strategy could also be interesting for resveratrol bioprocesses. Nevertheless, deletion of *ZWF1* was shown to have a positive impact on the production of muconic acid by forcing the carbon flux entry to the pentose phosphate pathway via transketolase,[8] which theoretically could also benefit resveratrol production, though this was later found to have a negative impact on yeast growth.[4] Therefore, careful selection of genetic modifications is crucial to avoid unnecessary metabolic burden and unexpected outcomes.

Due to the limited capacity of *S. cerevisiae* to produce malonyl-CoA compared to oleaginous microorganisms such as *Yarrowia lipolytica* and given the importance of this precursor in resveratrol biosynthesis, increasing its levels is crucial for higher resveratrol titres. The most used strategy to increase malonyl-CoA in resveratrol-producing *S. cerevisiae* strains relies on the overexpression of the double mutant *ACC1*$^{S659A, S1157A}$, encoding for the feedback-insensitive acetyl-CoA carboxylase.[5,6,9] A recent study showed that the expression of the homolog *ACC1* gene from *Y. lipolytica* in *S. cerevisiae* led to a higher accumulation of malonyl-CoA when compared to the overexpression of the native *ACC1* gene,[10] and this could also be interesting for increased resveratrol production. A high increase in resveratrol titre was also reported when multiple copies of the resveratrol biosynthetic pathway were introduced in the genome of *S. cerevisiae* [5,6] and *Y. lipolytica.*[11] In the latter, multiple copies of the biosynthetic pathway reduced the necessity of extensive engineering of the precursor supply. The proximity between enzymes in pathways involving multiple steps can also be relevant for increased resveratrol titres and bringing them together by fusion, scaffolding or compartmentalisation can be advantageous.[12] The fusion between 4CL ligase and STS has already been reported to highly improve resveratrol titres,[13] and the same rationale could be applied to other enzymes of the resveratrol biosynthetic pathway such as PAL (mainly cytoplasmatic) and C4H (attached to the endoplasmic reticulum), as it occurs in some plants to channel cinnamic acid.[14]

Vos et al.[15] reported on the necessity of decoupling cell growth and resveratrol production for industrial applications, as resveratrol biosynthesis is highly dependent on biomass formation but industrial processes in fed-batch reactors are generally implemented at low specific growth rates. Dynamic control

of the key intermediate malonyl-CoA using a biosensor to activate the expression of a heterologous pathway has been shown to have a high impact on 3-hydroxypropionic acid (3HP) production and could also be applied to other malonyl-CoA-derived products such as resveratrol.[16] Another study used a set of selected regulated promoters to enable the suppression of 3HP production when glucose is in excess in batch cultivation and its activation in glucose-limiting conditions, resulting in increased titres of this metabolite.[17]

Even though there is a lot to accomplish regarding the fine-tuning of the resveratrol biosynthetic pathway, developing efficient fermentation processes by thoroughly addressing the operation mode is mandatory in an industrial context. The implementation of fed-batch strategies has been associated with the highest resveratrol titres reported in the literature for *S. cerevisiae* [5,6] and other hosts like *Y. lipolytica.*[11] An interesting strategy for increased resveratrol titres should encompass the use of mixed feeding strategies such as glucose/ethanol, balancing yeast growth, which preferably occurs in glucose cultivation, and resveratrol production from ethanol, which is known to lead to higher resveratrol yields. This has already been reported to have a positive impact on the production of other compounds like geranylgeraniol,[18] also favoured by ethanol feed. In this sense, the integration of feedstocks with a high concentration of carbon sources, such as grape must (glucose and fructose) or wine lees (ethanol), could also be potentially interesting as feed solutions for fed-batch strategies, reducing the cost of the process. Harnessing on the insolubility of resveratrol in water, exploiting configurations like aqueous two-phase systems for an extractive fermentation can also be relevant, by coupling the aqueous fermentation medium with an organic phase, immiscible in water, with a high affinity for resveratrol (e.g. PEG 400). This was already reported to be beneficial for the biosynthesis of other chemicals like limonene[19] or vitamin A,[20] and would potentially solve two problems at once: product inhibition of fermentation process and simultaneous extraction of the product, facilitating downstream processing.

Coming to an end, the increasing demand for high-value chemicals such as resveratrol allied to the ever-growing environmental concern associated with fossil resources consumption is pushing the rapid expansion and establishment of biobased processes. While there is still much to accomplish in the path towards the implementation of agro-industrial residues in industrial bioprocesses for high-value low-volume chemicals, the outcomes of this thesis indicate that *S. cerevisiae* will most likely be a part of it, highlighting its versatility and crucial role in the development of sustainable processes and promoting a greener future towards a circular bioeconomy.

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Appendix

Chapter II

Table A2.1. List of plasmids used in the study.

Name	Parent plasmid	Biobricks	Description	Ref.
pCfB2312			Episomal plasmid for *Cas9* expression under *TEF1* promoter, KanMX selection marker	1
pCfB3035			EasyClone-MarkerFree Integrative Vector for X-4 insertion site (Chr X: 236336..237310)	2
pCfB3042			gRNA helper vector targeting X-4 insertion site, *NatMX* selection marker	2
pCfB2584			Multi-integrative plasmid, *Ty4*, *TDH3p-AtPAL2*, *FBA1p-AtC4H*, *PGK1p-At4CL2*, *TEF1p-VvVST1*, *KlURA3*	3
pCfB8531	pCfB3035	BB_CEC01	MarkerFree plasmid, X-4, *TDH3p-AtPAL2*, *FBA1p-AtC4H*, *PGK1p-At4CL2*, *TEF1p-VvVST1*	This study

Table A2.2. List of primers used in the study.

Name	Sequence (5' to 3')
PR-1551 (←At4CL2_rv)	CGTGCGAUTCAGTTCATCAAACCGTT
PR-1547 (AtC4H→_rv)	CACGCGAUTCAACAGTTTCTTGGCTT
PR-2221 (X4_up_conf_rv)	GTTGACACTTCTAAATAAGCGAATTTC
PR-905 (X4_up_conf_fw)	CTCACAAAGGGACGAATCCT
PR-2220 (X4_down_conf_fw)	CCTGCAGGACTAGTGCTGAG
PR-906 (X4_down_conf_rv)	GACGGTACGTTGACCAGAG
ID224 (ADH1_test_fw)	GAAATTCGCTTATTTAGAAGTGTC
PR-22847 (ADH1_C4L_seq_fw)	CGACCTCATGCTATACCT
PR-22848 (4CL_seq_int_rv)	TGTGTTCTTTGAGAGTTGGT
PR-22849 (4CL_seq_int_fw)	TGGTTTGGGATGTAGATATCTG
PR-22850 (4CL_seq__vst1_rv)	AAGTAGTAGTCGGCGTAATC
ID340 (PGK1_test_fw)	TACAGATCATCAAGGAAGTAATTATC
ID339 (TEF1_test_rv)	GCTCATTAGAAAGAAAGCATAGC
PR-22788 (VST1_seq_rv)	GCTCCTATTCCTATCTGCTG
PR-22851 (PAL2_int2_fw)	GCAAATACCGACCAAGAA
PR-22852 (PAL2_int3_fw)	TCACCAGAAGCAGTAATAG
PR-22790 (PAL2_int_seq_fw)	GGGTTAGCCAAGTATTGC
PR-22853 (FBA1_PAL2_seq_rv)	GAAGAGAACAGACAAATGG
PR-22789 (NAT5_PAL2_seq_fw)	GACGAAACCGAAAGTGATGATG
ID504 (tdh3UPfwd1)	TCTGCTGTAACCCGTACATG
PR-22854 (C4H_seq_fw)	ACGGTGAACATTGGAGAA
PR-22791 (FBA1_C4H_seq_fw)	CTCTTTCGGGCTCAATTG
PR-22792 (CYC1_C4H_seq_rv)	CCTGTTACTTCTTGCAGACATC

* Overhangs used for USER cloning are underlined.

Table A2.3. List of biobricks used in the study.

Biobrick	Description	Template	Forward primer	Reverse primer
BB_CEC02	RBP (*TDH3p-AtPAL2*, *FBA1p-AtC4H*, *PGK1p-At4CL2*, *TEF1p-VvVST1*)	pCfB2584	PR-1551	PR-1547

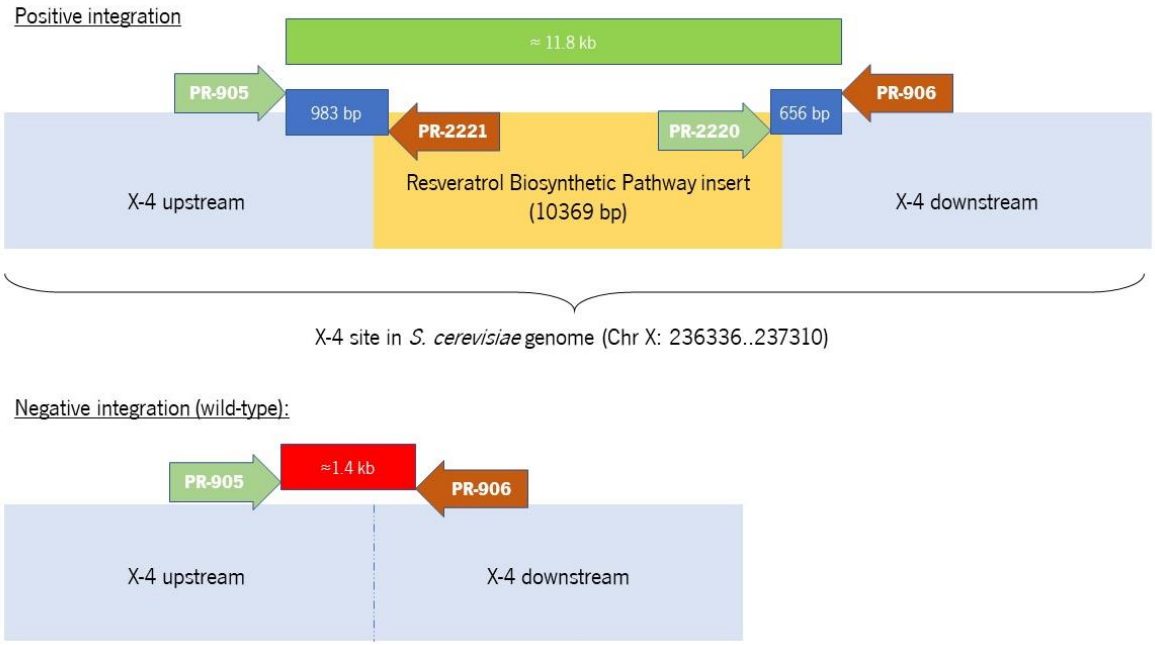

Figure A2.1. Representation of the confirmation of positive integration of the Resveratrol Biosynthetic Pathway in both alleles. For confirmation of integration in two copies, PR-905 and PR-906 were used (Table A2.2). This PCR reaction originates a band in both positive and negative (wild-type) integration cases. When integration occurs in both alleles of the chromosome, a single band around 11.8 kb is observed. When integration fails, a band of around 1.4 kb is observed (equal to the wild-type strain). When integration occurs only in one allele (one copy), both bands (1.4 kb and 11.8 kb) are observed. The first situation (a single 11.8 kb band) was observed for all strains studied, hereby showing integration of two copies of the Resveratrol Biosynthetic Pathway.

Chapter IV

Table A4.1. List of plasmids used in the study.

Name	Parent plasmid	Cloned biobricks	Description	Ref.
pCfB2312			Episomal plasmid for Cas9 expression under *TEF1* promoter, *KanMX* selection marker	1
pCfB3042			gRNA helper vector targeting X-4 insertion site, *NatMX* selection marker	2
pCfB3049			gRNA helper vector targeting XII-4 insertion site, *NatMX* selection marker	2
pCfB3040			EasyClone-MarkerFree Integrative Vector for XII-4 insertion site (Chr XII:830227..831248)	2
pCfB2767			Multi-integrative plasmid, Ty4, P_{TDH3}-*AtPAL2*, P_{FBA1}-*AtC4H*, P_{PGK1}-*At4CL2*, P_{TEF1}-*VvVST1*, *KlURA3*	3
B430	pCfB3040	BB_CEC02	MarkerFree plasmid, X-4, P_{TDH3}-*AtPAL2*, P_{FBA1}-*AtC4H*, P_{PGK1}-*At4CL2*, P_{TEF1}-*VvVST1*	This study

Table A4.2. List of primers used in the study.

Name	Sequence (5' to 3')
PR-1496 (CYB5/ATR2_fw)	CGTGCGAUTCATTCGTTCAACAAATAATAAGC
PR-1555 (CYB5/ATR2_rv)	CACGCGAUTCACCAGACATCTCTCAA
PR-2221 (universal_up_conf_rv)	GTTGACACTTCTAAATAAGCGAATTTC
PR-2220 (universal_down_conf_fw)	CCTGCAGGACTAGTGCTGAG
PR-905 (X-4_up_conf_fw)	CTCACAAAGGGACGAATCCT
PR-906 (X-4_down_conf_rv)	GACGGTACGTTGACCAGAG
PR-897 (XII-4_up_conf_fw)	GAACTGACGTCGAAGGCTCT
PR-898 (XII-4_down_conf_fw)	CGTGAAATCTCTTTGCGGTAG
ID224 (ADH1_test_fw)	GAAATTCGCTTATTTAGAAGTGTC
PR-22847 (ADH1_C4L_seq_fw)	CGACCTCATGCTATACCT
PR-22848 (4CL_seq_int_rv)	TGTGTTCTTTGAGAGTTGGT
PR-22849 (4CL_seq_int_fw)	TGGTTTGGGATGTAGATATCTG
PR-22850 (4CL_seq__vst1_rv)	AAGTAGTAGTCGGCGTAATC
ID340 (PGK1_test_fw)	TACAGATCATCAAGGAAGTAATTATC
ID339 (TEF1_test_rv)	GCTCATTAGAAAGAAAGCATAGC

Table A4.2. (continued)

Name	Sequence (5' to 3')
PR-22788 (VST1_seq_rv)	GCTCCTATTCCTATCTGCTG
PR-22851 (PAL2_int2_fw)	GCAAATACCGACCAAGAA
PR-22852 (PAL2_int3_fw)	TCACCAGAAGCAGTAATAG
PR-22790 (PAL2_int_seq_fw)	GGGTTAGCCAAGTATTGC
PR-22853 (FBA1_PAL2_seq_rv)	GAAGAGAACAGACAAATGG
PR-22789 (NAT5_PAL2_seq_fw)	GACGAAACCGAAAGTGATGATG
ID504 (tdh3UPfwd1)	TCTGCTGTAACCCGTACATG
PR-22854 (C4H_seq_fw)	ACGGTGAACATTGGAGAA
PR-22791 (FBA1_C4H_seq_fw)	CTCTTTCGGGCTCAATTG
PR-22792 (CYC1_C4H_seq_rv)	CCTGTTACTTCTTGCAGACATC
ID18 (CYC1_rv)	CACGCGTCTGTACAGAAA
PR-22857 (ATR2_seq_int_fw)	GTTACACCGTTTTTGATGCT

* Overhangs used for USER cloning are underlined.

Table A4.3. List of biobricks used in the study.

Biobrick	Description	Template	Forward primer	Reverse primer
BB_CEC02	*ScCyb5←PGK1p - TEF1p→AtATR2*	pCfB2767	PR-1496	PR-1555

Table A4.4. List of yeast strains used in the study.

Yeast strain	Parent strain	Relevant genotype	Ref.
L323 (also reported as Ethanol Red RBP)	Ethanol Red®	*X-4: TDH3p→AtPAL2, FBA1p→AtC4H, PGK1p→At4CL2, TEF1p→VvVST1*	5
XylC2 V1 (ST5415)	Ethanol Red®	X-3: *TEF1p→RKI1, TDH3p→RPE1* XI-3: *TEF1p→TKL1, TDH3p→PsTAL1* XII-2: *TEF1p→CpXylA, TDH3p→PsSUT1* XII-5: *TEF1p→CpXylA, TDH3p→PsXYL3* *ΔGRE3::TEF1p→CpXylA/ ΔGRE3::TEF1p→CpXylA*	6
L326	XylC2 V1 (ST5415)	*X-4: TDH3p→AtPAL2, FBA1p→AtC4H, PGK1p→At4CL2, TEF1p→VvVST1* X-3: *TEF1p→RKI1, TDH3p→RPE1* XI-3: *TEF1p→TKL1, TDH3p→PsTAL1* XII-2: *TEF1p→CpXylA, TDH3p→PsSUT1* XII-5: *TEF1p→CpXylA, TDH3p→PsXYL3* *ΔGRE3::TEF1p→CpXylA/ ΔGRE3::TEF1p→CpXylA*	This study
L543	L326	XII-4: *ScCyb5←PGK1p - TEF1p→AtATR2* *X-4: TDH3p→AtPAL2, FBA1p→AtC4H, PGK1p→At4CL2, TEF1p→VvVST1* X-3: *TEF1p→RKI1, TDH3p→RPE1* XI-3: *TEF1p→TKL1, TDH3p→PsTAL1* XII-2: *TEF1p→CpXylA, TDH3p→PsSUT1* XII-5: *TEF1p→CpXylA, TDH3p→PsXYL3* *ΔGRE3::TEF1p→CpXylA/ ΔGRE3::TEF1p→CpXylA*	This study

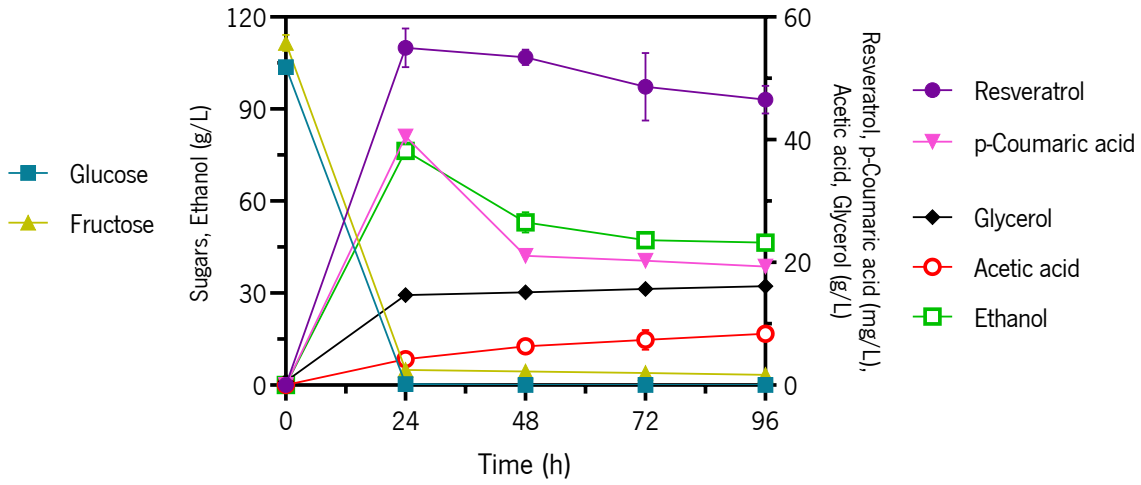

Figure A4.1. Fermentation profile of media with 95% of grape must dissolved supplemented with 7.5 g/L of yeast extract.

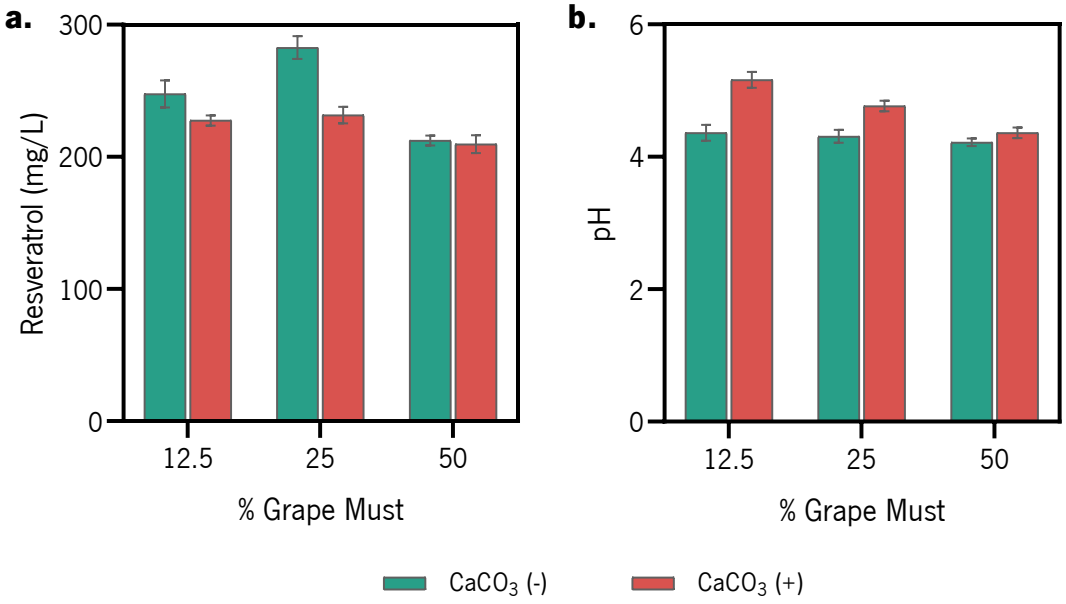

Figure A4.2. Difference between fermentation without ($CaCO_3$ (-)) and with ($CaCO_3$ (+)) of different concentrations of grape must dissolved in the fermentation media, from 12.5 to 50%, supplemented with 7.5 g/L of yeast extract.

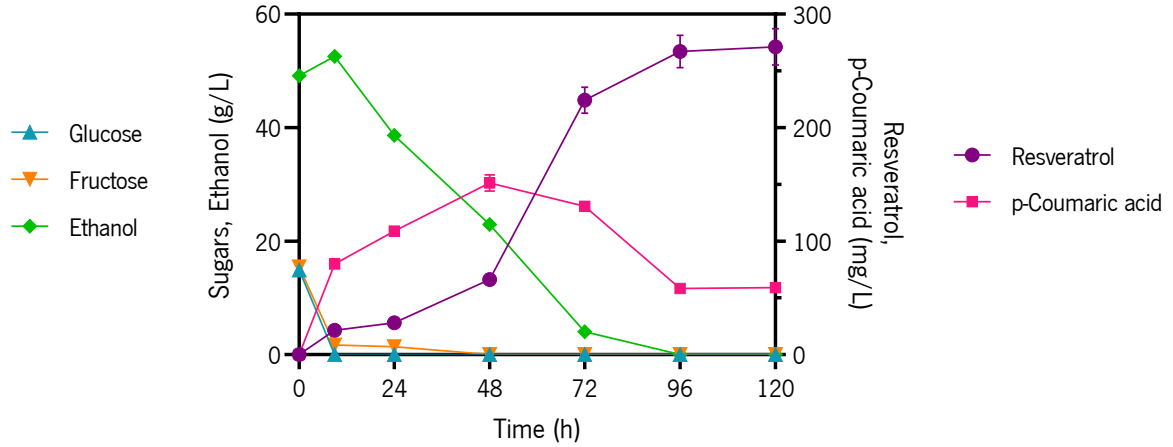

Figure A4.3. Fermentation profile of media with 50% of wine lees together with 10% of grape must dissolved.

DNA sequences

DNA sequence of the Resveratrol Biosynthetic Pathway (*TDH3p→AtPAL2, FBA1p→AtC4H, PGK1p→At4CL2, TEF1p→VvVST1*) used in this thesis (9955 bp)

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DNA sequence of *PGK1p→ScCyb5, TEF1p→AtATR2* used in this thesis (3933 bp)

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AAACTTCTGGTAGATACTTGAGAGATGTCTGGTGA

DNA sequence of the lactose metabolic pathway used in this thesis (*TEF1p→ KlLAC4, PGK1p→KlLAC12*) (6257 bp)

AAATGTCTTGCCTTATTCCTGAGAATTTAAGGAACCCCAAAAAGGTTCACGAAAATAGATTGCCTACTAGGGCTT
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