Thesis for the Degree of Doctor of Philosophy

Mapping Phenolics Metabolism and Metabolic Engineering of *Saccharomyces cerevisiae* for Increased Endogenous Catabolism of Phenolic Compounds

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Cover illustration: A simplified conversion route for coniferyl aldehyde in *Saccharomyces cerevisiae* proposed in this thesis.
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Abstract

Sustainable, biotechnological utilization of non-food, plant biomass has been demonstrated to be a viable means of producing energy, fuels, materials and chemicals, representing a paradigm shift from fossil-derived sources. However, the presence of chemicals that inhibit fermentation by microorganisms such as *Saccharomyces cerevisiae*, commonly used for bioconversion, causes a bottleneck in such processes. Phenolic compounds are aromatic compounds that serve as building blocks of lignin in plants. During the deconstruction of plant biomass, phenolic compounds are released as degradation products from the lignin component of wood into the hydrolysates, inhibiting fermentation. The aim of the work presented in this thesis was to explore approaches for the development of strains of *Saccharomyces cerevisiae* that have improved tolerance to phenolic compounds, and to better understand its endogenous metabolism of phenolic compounds. A study was performed on the interaction between the yeast and phenolic compounds using single phenolic compounds in defined growth medium. The toxicity of thirteen phenolic compounds was determined. The concentrations at which each compound completely inhibited the growth of *S. cerevisiae* was found to differ among the compounds, and three distinct physiological responses were observed. The influence of the structure and the presence of the methyl, aldehyde, carboxylic acid and hydroxyl functional side groups that often decorate phenolic compounds were studied in coniferyl aldehyde, ferulic acid and *p*-coumaric acid. The conversion of these compounds into less toxic phenolic compounds was observed. Based on the product profile, a conversion route was hypothesized for the catabolism of phenolic compounds in *S. cerevisiae*. Finally, two strains of *S. cerevisiae*, *B_CALD* and *APT_1*, were engineered. *B_CALD* was metabolically engineered to exhibit increased endogenous conversion of coniferyl aldehyde, while *APT_1* was metabolically engineered to exhibit increased endogenous conversion of coniferyl aldehyde, ferulic acid and p-coumaric acid, and to test the hypothesized conversion pathway. The engineering of both *B_CALD* and *APT_1* was successful.

Keywords: Phenolic compounds, inhibitor, toxicity, conversion, *Saccharomyces cerevisiae*

List of publications

The following papers are included in this thesis are referred to in the text by their Roman numerals

I. Adeboye PT, Bettiga M, Olsson L. The chemical nature of phenolic compounds determines their toxicity and induces distinct physiological responses in *Saccharomyces cerevisiae* in lignocellulose hydrolysates. AMB Express. 2014;4:46.

II. Adeboye PT, Bettiga M, Aldaeus F, Larsson P, Olsson L. Catabolism of coniferyl aldehyde, ferulic acid and p-coumaric acid by *Saccharomyces cerevisiae* yields less toxic products. Microbial cell factories. 2015;14(1):149.

III. Adeboye PT, Olsson L, Bettiga M: A coniferyl aldehyde dehydrogenase gene from *Pseudomonas sp*. strain HR199 enhances the conversion of coniferyl aldehyde by *Saccharomyces cerevisiae*. Bioresource Technol 2016, 212:11-19.

IV. Adeboye PT, Bettiga M, Olsson L. *ALD5, PAD1, ATF1* and *ATF2* facilitate the catabolism of coniferyl aldehyde, ferulic acid and *p*-coumaric acid in *Saccharomyces cerevisiae.* Submitted for publication.

I designed and performed the experiments, analysed the data and wrote the Papers I-IV in this thesis.

Preface

The work described in this PhD thesis was carried out according to the requirements for a Doctoral Degree at the Department of Biology and Biological Engineering, Chalmers University of Technology, Sweden. The work is primarily focused on the development of *Saccharomyces cerevisiae* with improved conversion of, and tolerance to, phenolic compounds. The work was carried out under the supervision of Professor Lisbeth Olsson and Associate professor Maurizio Bettiga.

This PhD project was initiated in June 2011 as part of a collaboration between Innventia AB, Stockholm and the Industrial Biotechnology Group at Chalmers. Some GC-MS analyses were performed in collaboration with staff at Innventia AB. This project work was funded by the Swedish Research Council (Vetenskapsrådet) under grant no. 621-2010-3788, under the Programme for Strategic Energy Research.

Adeboye Peter Temitope

June, 2016

List of figures and tables

Figures

Tables

ABBREVIATIONS AND SYMBOLS

GC-MS	Gas chromatography mass spectrometry
CALDH	Coniferyl aldehyde dehydrogenase
YMMM	Yeast minimal mineral medium
ALD5	Aldehyde dehydrogenase 5
PAD1	Phenylacrylic acid decarboxylase
ATF1	Alcohol acetyltransferase 1
ATF2	Alcohol acetyltransferase 2
HPLC	High pressure liquid chromatography

Table of contents

Introduction

Humans have always been dependent on nature for survival, and plants have always been an integral part of our existence, from building shelters to making fires, food and elixirs. Microorganisms have also played a vital role in our history. One of these microorganisms is baker's yeast, *Saccharomyces cerevisiae*, which is commonly cited as being the first microorganism used by man [1]. *S. cerevisiae* is a natural agent in microbial decay and fermentative activity that take place widely in nature, producing ethanol and carbon dioxide [2]. It has therefore been used for several millennia to make fermented beverages and bread [3, 4]. Indeed, the name *cerevisiae* originates from the Gaelic word *kerevigia* and the old French word *cervoise* which both mean "beer" [5]. Being eukaryotic, yeast has also served as a cellular model in many scientific studies [6]. In this thesis, the terms yeast and *S. cerevisiae* are used interchangeably.

The use of liquid biofuels such as bioethanol in which yeast is very relevant, predates the use of fossil fuels such as petrol and diesel [7]. The early 20th century saw the use of cars powered by ethanol derived from hemp, and the famous inventor Henry Ford was quoted as saying, "The fuel of the future…is going to come from fruit like that sumac out by the road, or from apples, weeds, sawdust – almost anything. There is fuel in every bit of vegetable matter that can be fermented" [8]. However, cheap fossil fuel reduced the demand for bioethanol [9]. Environmental concerns and dwindling resources have led to increased demands for bioethanol once again, but from more sustainable sources. First generation bioethanol was derived from edible agricultural biomass such as cassava, soybean, sugarcane, sugar beet, and food grains such as wheat, barley, rye, or sweet sorghum [10, 11]. However, this was deemed unsustainable due to scarce resources, resulting from drought and the limited availability of arable land. Therefore, second generation ethanol is based on lignocellulosic materials that do not compete with food supplies [12]. The desire for cost-effective, cleaner processes and reduced waste in the forest industry has also driven the biorefinery concept, in which forest industries make use of residues and side streams for conversion into useful resources [13-15].

Although bioethanol derived from lignocellulosic biomass has great potential for sustainable industrial biofuel production, the recalcitrance of the biomass is a significant

problem. Harsh pretreatment processes are needed to deconstruct the biomass and make it accessible for bioconversion. A major consequence to this pretreatment is the release of degradation products such as organic acids, furaldehydes and phenolic compounds, which are inhibitory to the microorganisms and enzymes used for bioconversion. Also, forest-dependent industries, such as the pulp and paper industry, are primarily interested in the cellulosic part of wood, and their pretreatment processes are tailored towards retaining the bulk of the cellulose while removing the hemicellulose and lignin fractions in side streams. This means that the side streams are poor in fermentable sugars, while having a high concentration of phenolic compounds from the depolymerized lignin.

The aim of this work was to investigate the possibility to utilize side streams derived from softwood (spruce) in the pulping industry for the production of second generation biofuel and biochemicals. The fermentability of spruce pulping side streams, was therefore investigated, they were found to be poorly fermentable as they are rich in phenolic compounds. Attention was subsequently focused on understanding the influence of phenolic compounds on yeast, and how yeast performs in the presence of phenolic compounds (Paper I). It was found that the endogenous catabolism of phenolic compounds led to *in situ* detoxification of phenolic compounds through a process in which the phenolic compounds are converted into less inhibitory compounds by the yeast (Paper II). Finally, yeast strains with increased capability for the bioconversion of phenolic compounds were developed (Paper III and Paper IV). An illustration of the work described in this thesis is shown in Figure 1 below.

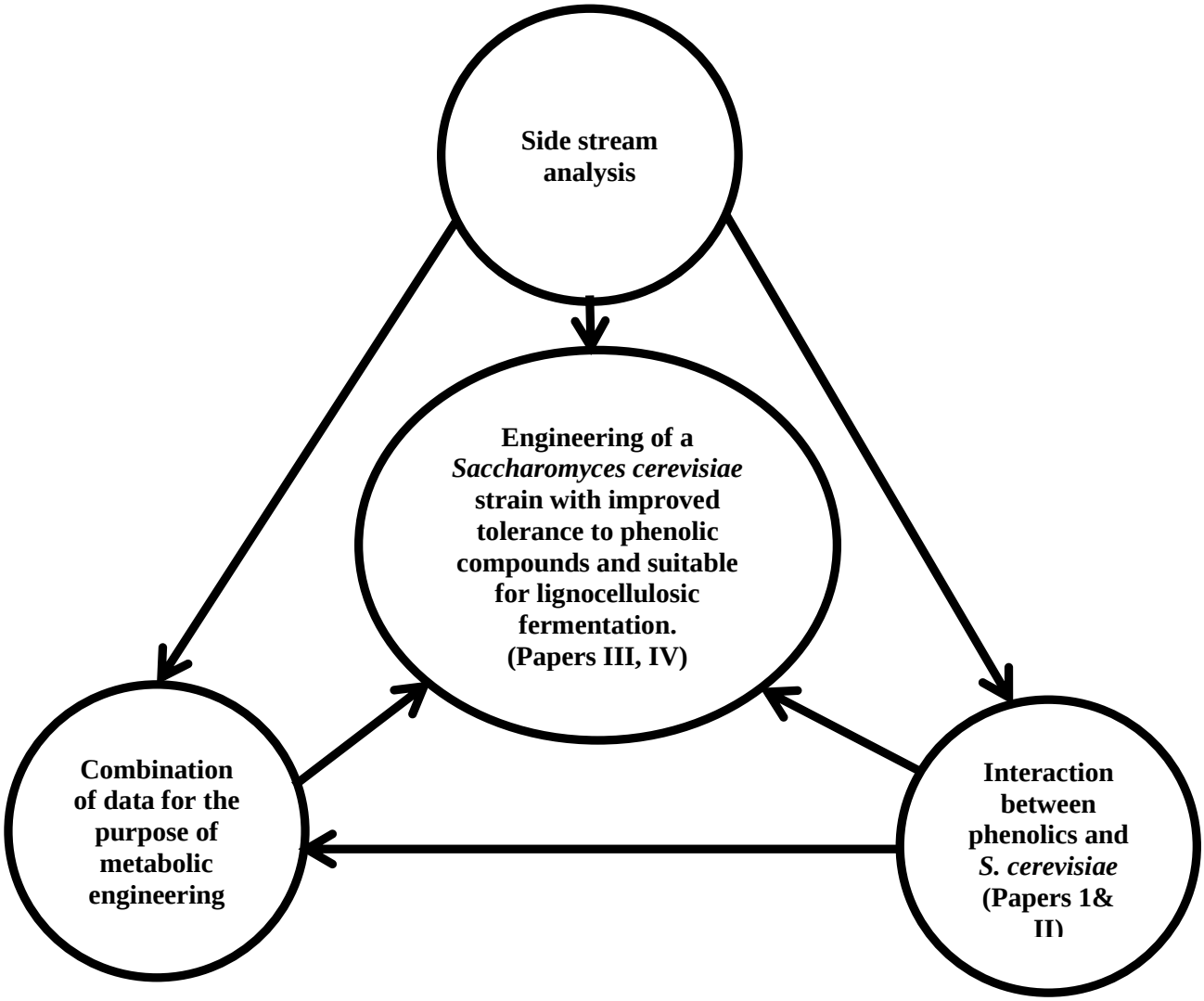

Figure 1: A schematic description of the outline and strategies of work described in this thesis.

Four peer-reviewed articles and a patent application resulted from the work presented in this thesis. Paper I reports on the physiological influence of thirteen phenolic compounds on yeast. The compounds were selected based on the phenolics profile in spruce-derived hydrolysates and side streams. This study also revealed that different phenolic compounds have different concentration thresholds at which they affect yeast, and that their functional side groups tend to influence their degree of inhibition. The second study reported in Paper II, focused on the investigation of the catabolism of coniferyl aldehyde, ferulic acid and *p*-coumaric acid by yeast. Based on the results, a conversion route for these three and it was also hypothesized that this route is similar for other phenolics. In the third study, a yeast strain called *B_CALD*, exhibiting improved endogenous conversion of coniferyl aldehyde, was engineered by heterologous expression of coniferyl aldehyde

dehydrogenase (*CALDH*) from *Pseudomonas* and is presented as a proof of concept. The third study in reported in Paper III. In the fourth and final study, it was hypothesized that *ALD5, PAD1, ATF1* and *ATF2* played significant roles in the catabolism of phenolic compounds in yeast. The proposed conversion pathway with the four suggested enzymes was engineered in a new yeast strain, *APT_1*, which exhibited an improved ability to convert coniferyl aldehyde, ferulic acid and p-coumaric acid. The conversion of coniferyl aldehyde, ferulic acid and p-coumaric acid by the recombinant strain and a control strain was monitored over time. The conversion products (metabolites) were identified and quantified using GC-MS, and most of them were found to be transient. Paper IV provides extensive results on the catabolism of coniferyl aldehyde, ferulic acid and p-coumaric acid. An elaborate metabolic route for the three compounds in yeast is also proposed, based on the quantification of the metabolites. The study reported in paper IV is also the subject of the patent application.

The challenges facing second generation biofuel and biochemical production, including the problems associated with using pulping side streams, the inhibitory effect of phenolic compounds, the bioconversion of phenolic compounds and their potentials are discussed in this thesis. Modern trends and strategies in metabolic engineering to increase the tolerance of yeast to phenolic compounds are also discussed. The engineering strategies used in the present work to develop two recombinant strains are then described, and the challenges and prospects of developing a *S. cerevisiae* strain that can catabolize compounds and tolerate phenolic compounds better than the presently available *S. cerevisiae* strains are discussed.

CHAPTER 2: Second generation ethanol: The role of substrates and *Saccharomyces cerevisiae*

Second generation biofuels and biochemicals are derived from lignocellulosic, non-food, forest and agricultural crop residues [16]. The production of biofuel started more than a century ago with the bioconversion of starchy substrates such as corn, potatoes and sugar beet to ethanol. However, this competed with food supplies and was generally considered unsustainable [16]. Several problems undermined the production of first generation biofuels, such as a reduction in oil prices, competition for land and water for food production, and changes in government policies [16-18]. Lignocellulosic biofuels have economic, strategic and environmental advantages over food-based bioethanol, and have therefore become increasingly favoured over first generation biofuels [19, 20].

The conversion of lignocellulosic biomass into the desired end products is a technically demanding process [21, 22]. The woody nature and generally, the physical composition of lignocellulosic biomass require a series of processes, starting with pretreatment to deconstruct the biomass [23, 24], followed by other processes to obtain the desired products. The process technology for the conversion of lignocellulosic biomass to bioethanol and other chemicals has advanced over the decades, and an increasing number of products are being derived from lignocellulose [25] (Figure 2). The processes involved in the microbial conversion of lignocellulosic biomass rely heavily on the use of mechanical and chemical energy to make the lignocellulosic biomass accessible to the microorganisms employed as biocatalysts for conversion into the desired products.

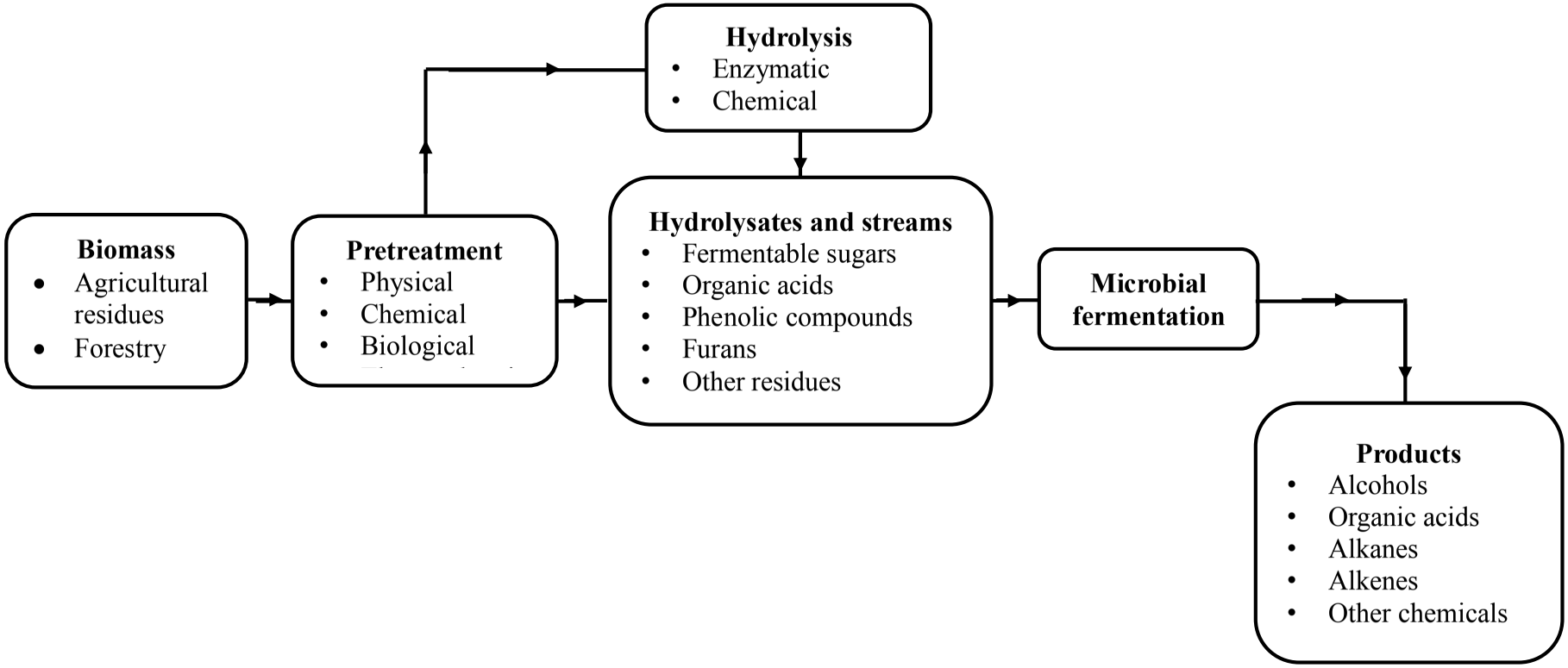

Figure 2: Schematic illustration of the processes involved in the conversion of lignocellulosic biomass into second generation biofuel and chemicals.

2.1 Substrate pre-treatment

The pretreatment of lignocellulosic biomass is the first step involved in deriving the desired products from lignocellulosic biomass (Figure 2). Plant cell walls are structurally made up of microfibrils of crystalline cellulose, hemicelluloses and lignin sheath in a lignin carbohydrate complex, all tightly bound in a network of intra- and inter-molecular hydrogen bonds [26, 27]. The physicochemical structure makes it difficult for enzymes such as cellulases to bind onto the surface of cellulose molecules, and to act on the specific chemical bonds they target. Mechanical force is usually first applied to reduce the biomass into 10-30 mm particles, after which pretreatment is applied to deconstruct the lignin–carbohydrate complex for subsequent enzymatic hydrolysis of cellulose [28, 29]. In general, the main goal of pretreatment is to increase the accessibility and digestibility of biomass in order to facilitate the release of the maximum amount of fermentable sugars. The method of pretreatment therefore often depends on the nature of the biomass and the type of products desired [29, 30]. Pretreatment may be physical [31], chemical, e.g., ionic liquids and acids [29, 32-34], biological [35-37] or thermochemical [38, 39]. Thermochemical biomass pretreatment in which heat is combined with either an acid or an alkaline is the most common of the pretreatment methods.

In a biorefinery concept in which the cellulose in the biomass is intended for a different purpose other than fuel production, for example, in a pulping mill, a different method of pretreatment is usually applied [40]. The tons of lignocellulosic waste produced by agricultural and forest-dependent industries are potential sources of energy that can replace fossil-based fuels and chemicals [35, 41, 42]. After the biomass has been subjected to pulping, the pulping side streams are sometimes channelled towards bioconversion to bioethanol or other chemicals [43]. The use of pulping streams for the production of bioethanol or other chemicals is beneficial in a biorefinery concept, where environmental concerns and waste reduction are important [44, 45]. Innventia AB our project partner in this work, is involved in research in the field of biorefineries and has detailed knowledge in pulping and the production of cellulose for various industrial applications. Three side streams, prehydrolysate, black liquor and the oxygen delignification stream, which will be discussed in later chapters, were supplied by Innventia AB. Since pulping technologies on pilot scale constitute part of the core

competence at Innventia AB, and the three side streams used in this project were derived from a pulping process, it is worthwhile to briefly discuss the methodology of pulping.

2.2 The pulping process

Pulping is the process of physically and or chemically breaking down wood into discrete fibres known as pulp [46, 47]. The aim of pulping is to liberate cellulose fibres from the lignin and hemicellulose components of wood or other raw material, leaving the cellulose mostly intact for further usage such as paper manufacture [48]. Pulping is a well-established and popular technology for biomass disintegration to make wood pulps [49]. There are different types of pulping processes, and the choice of method is dependent on the type of raw material and the kind of pulp required for paper making [48]. Chemical pulping is a widespread process. The four classical methods used in chemical pulping are the kraft, sulphite, soda and neutral sulphite semi-chemical pulping processes [50]. Pulping involves cooking wood biomass to obtain cellulose fibres, during which delignification takes place and monomeric sugars are released from the hemicellulose fraction into the cooking liquor [48]. The cooking liquor is then released as the process streams. Cooking liquor such as spent sulphite liquor, black liquor, the delignification stream and pulp residues, are useful sources of energy and lignin, as well as having the potential for several applications, including bioethanol and chemical production [51]. The main component of wood that needs to be removed before the wood can be processed into paper and other cellulosic products is lignin. Lignin itself is a natural, heterogeneous polymer responsible for the structural rigidity of cells and tissues, and is essential to the vascular development in plants [52, 53]. It is mainly made up of phenylpropane units derived from guaiacol, p-hydroxyphenol and syringol, all interconnected in a C-C bond [54, 55]

The pulping streams used in the present work were produced by Innventia AB employing an alkaline-based process called soda pulping. This is a seven-step process, illustrated in Figure 3. Wood is first debarked and cut into small chips. The chips are then treated with superheated steam, leading to autohydrolysis, which also opens up the wood matrix [56]. The prehydrolysate side stream is derived from this process. Delignification is achieved during soda cooking, where the hydroxide plays the most important role in the delignification process. After this stage, the cooking liquor, commonly known as black

liquor [57, 58], is removed. The pulp is thoroughly washed and defibrillated, and the lignin residue remaining in the pulp is removed by oxygen delignification [58].

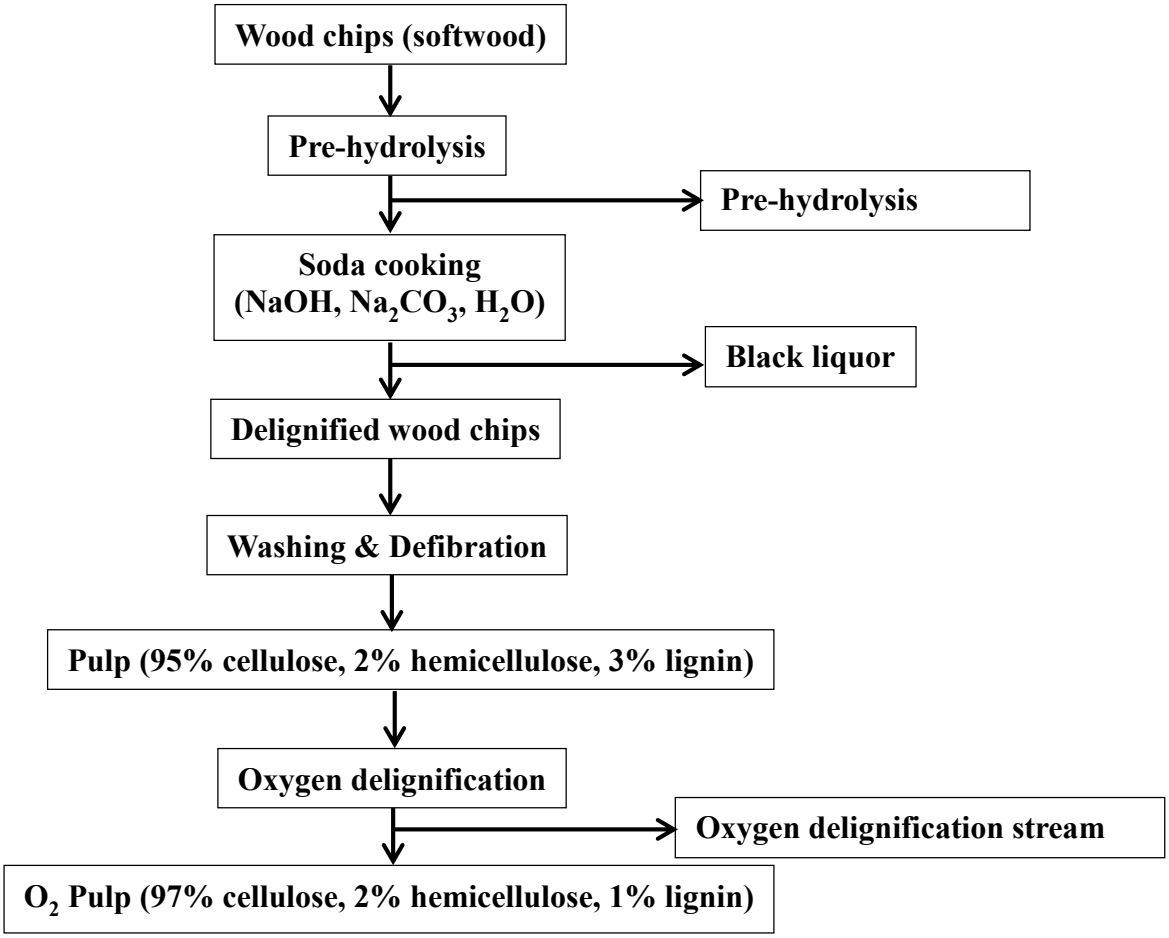

Figure 3: wood pulping process generating lignocellulosic side streams at Innventia.

2.3 Substrate composition

The microbes used to ferment a substrate or side stream utilize monosaccharides as a source of carbon and energy. Other components in the substrate stream may be used as a source of nutrition, or may interfere with the microbial conversion. In order to determine the fermentability of a stream it is necessary to investigate its composition. Compositional analysis was therefore carried out on the three streams used in this work. Lignocellulosic substrates are often rich in inhibitory compounds such as furans, organic acids and phenolic compounds, which are derived from the depolymerization of wood cellulose, hemicellulose and lignin polymers. During pretreatment, the hemi-cellulose, which is a heterogeneous polymer, is usually degraded into products such as pentose and hexose sugars, and sugar acids [59]. Aliphatic acids consisting mainly of acetic acid, formic acid

and levulinic acid, as well as 5-hydroxymethylfurfural (HMF) and furfural are also formed [59, 60]. Phenolic compounds are usually derived from lignin [60]. As the three pulping side streams used in this study were products of a process designed to remove lignin from wood, they were very rich in depolymerized hemicellulose and lignin residues. The most abundant compounds were phenolic compounds derived from lignin (Table 1). Lignin consists mainly of aromatic compounds, and the structure varies with the structure of the plant [52, 61, 62], explaining why the streams were very rich in phenolic compounds.

Table 1: Composition of the prehydrolysate, black liquor, and oxygen delignification side stream obtained from soda pulping, at the initial pH, and when the pH was adjusted to 5

	Prehydrolysate		**Black liquor**		**Oxygen delignification**	
	Initial pH 3.0	**pH 5.0**	**Initial pH 12.4**	**pH 5.0**	**Initial pH 11.8**	**pH 5.0**
	(g/l)	(g/l)	(g/l)	(g/l)	(g/l)	(g/l)
Arabinose	0.69	0.57	0.73	0.32	0.41	0.43
Galactose	0.92	0.74	0.62			
Glucose	0.46	0.37	0.26	0.62		0.30
Xylose	1.22	1.02				0.26
Mannose	0.99	0.83	0.00			
Formic acid	0.05	0.05	0.17	0.42	0.04	0.05
Acetate	0.72	0.62	0.47	1.41	0.07	0.05
HMF	0.75	0.36	0.04			
Furfural	1.50	0.66				
Total phenols	2.25	1.21	20.17	3.68	0.56	0.52

The sugars, acids and furans were measure using HPLC, phenolic compounds were measure using GC-MS and Folin–Denis reagent [63]

Apart from being rich in phenolics and low in fermentable sugars, the side streams were not suitable for fermentative microorganisms such as *S. cerevisiae* due to their pH values. The black liquor and oxygen delignification stream were derived from the alkaline pulping process, while the prehydrolysate was obtained after autohydrolysis. The pH

values for the prehydrolysate, black liquor and oxygen delignification stream were 3.0, 12.4 and 11.8, respectively. The pH was adjusted to pH 5.0, in order to reduce the phenolic compounds in the side streams and to make the liquor more suitable for the growth of *S. cerevisiae*. After pH adjustment, the total phenolic contents of the side streams were significantly reduced. In black liquor in particular, the total phenolic content was reduced to 18% of its original concentration (Table 1). The phenolic contents were significantly reduced as the phenolic compounds were derived from dissolved lignin, which was mostly alkali-soluble lignin. Upon decreasing the pH, repolymerization occurs, and the lignin is precipitated as soda lignin together with a high amount of NaCl, as the pH was adjusted with 2M HCl.

2.4 Fermentability of substrates, prehydrolysate, black liquor and oxygen delignification streams as case studies

The usefulness of a lignocellulosic substrate for bioconversion into second generation biofuel and biochemicals depends largely on its fermentability, and the aim of biomass pretreatment is thus to make the biomass more accessible to enzymes and micro-organisms for bioconversion. One of the challenges of pretreatment has been finding a balance between a substrate that is sufficiently pretreated, while releasing the minimum amount of inhibitors [30, 64, 65]. For this reason, the fermentability of the three side streams from the soda cooking process was determined by cultivating *S. cerevisiae* in them. The streams were supplemented with 20 g/L glucose. A reference cultivation was performed in yeast minimal mineral medium (YMMM) [66], and a second reference cultivation? was performed in an inhibitor cocktail composed of known inhibitors in softwood hydrolysates [67]. The industrial *S. cerevisiae* strain Ethanol Red® was used. The screening cultivation was performed in Erlenmeyer flasks, and the results are illustrated in Figure 4. Normal growth, and a maximum specific growth rate of 0.22 ± 0.02 h^{-1} was observed in *S. cerevisiae* Ethanol Red® in YMMM and 0.16 ± 0.11 h^{-1} in cultivations in the inhibitor cocktail, while specific growth rates of 0.05 ± 0.02 h^{-1}, 0.04 ± 0.01 h^{-1} and 0.07 ± 0.02 h^{-1} were observed in the cultivations in prehydrolysate, black liquor and the oxygen delignification stream, respectively. This screening experiment demonstrated the inhibitory capacity and non-fermentability of the three side streams.

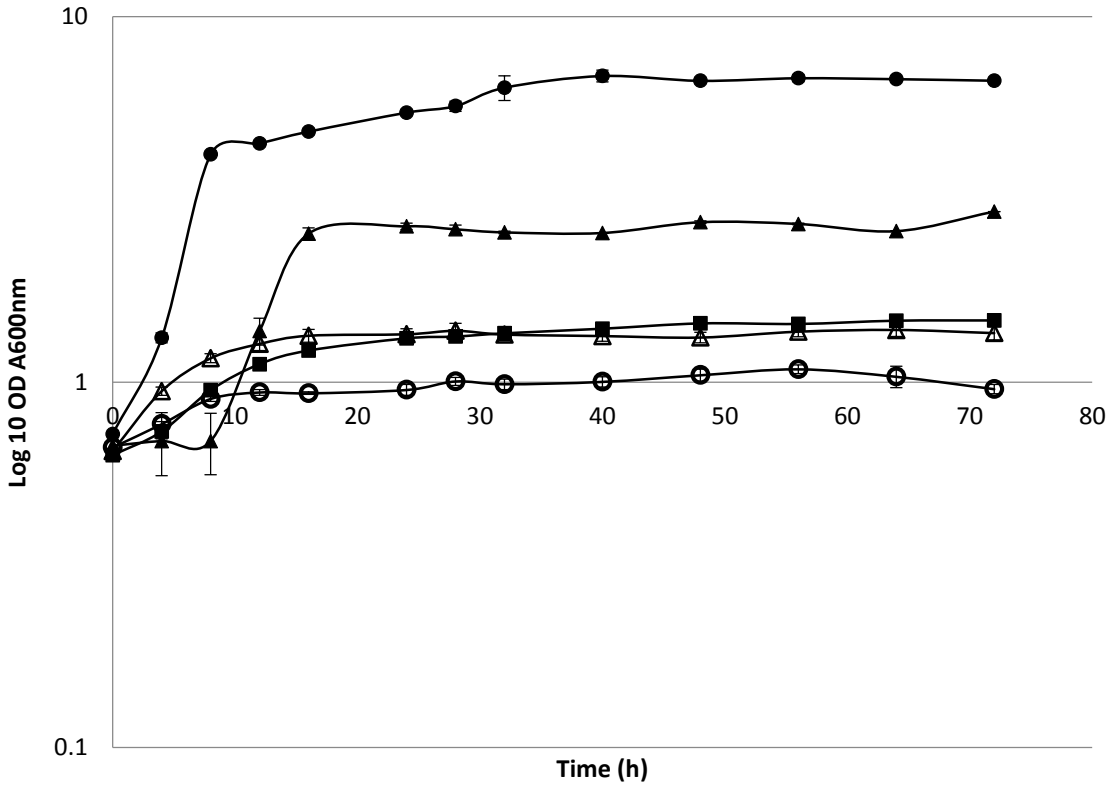

Figure 4: Fermentability of prehydrolysate, black liquor and oxygen delignification streams from pulping process using *S. cerevisiae* strain Ethanol Red®.

S. cerevisiae in: YMMM medium (●), inhibitor cocktail (▲), black liquor (⊙), prehydrolysate stream (■) and oxygen delignification stream (▲)

The non-fermentability of the substrates strongly correlated with the presence of a significant amount of phenolic compounds in the side streams. This gave rise to the conclusion that the non-fermentability of the streams is due to the presence of phenolic compounds.

During pretreatment, a diverse array of phenolic compounds are released into the hydrolysates from the depolymerization of lignin [68, 69]. The phenolic profiles of hydrolysates vary, depending on the pretreatment method and the nature of the biomass [60]. The phenolic compounds act together with other inhibitors present in the hydrolysates to hinder the bioconversion of the hydrolysates [70-72]. Due to the variety of phenolic compounds present in hydrolysates, it is impossible to delineate the inhibitory activity of individual phenolic compounds from that of other phenolic and inhibitory compounds in the liquid. Therefore, single phenolic compounds were used in a defined

medium to study the inhibitory role of individual phenolic compounds on *S. cerevisiae*. The effect of individual phenolic compounds on the physiology of *S. cerevisiae*, the effect of the concentration of the compound, possible effects of the structure of the compound on its activity, and the effects of phenolic stress on *S. cerevisiae* were deemed to be of paramount importance, and were therefore studied further. In addition, the fascinating question of whether substrates that contain poorly fermentable sugars but are rich in other compounds, such as phenolics, could be useful in the production of other chemicals by microbial conversion was also investigated.

2.5 *S. cerevisiae* as a microbial workhorse for science and industry

Until now, substrates have been discussed, however, the most suitable substrate requires the right biocatalyst to convert it into the desired product. In this work, *S. cerevisiae* was the biocatalyst of interest. *S. cerevisiae*, known more commonly as baker's yeast, and is the most widely studied of the eukaryotic microorganisms [73]. Yeast has been a workhorse for the production of various products in the food, pharmaceutical, chemical and energy industries [74]. Although it has been widely used for the production of ethanol for more than a century, and is known to be tolerant to harsh growth conditions [74], it has been shown that performance varies between strains in lignocellulosic fermentation [75]. It is therefore important to select a strain of *S. cerevisiae* that is well suited for lignocellulosic fermentation. One of the advantages of *S. cerevisiae* is the vast amount of knowledge available from decades of research on its physiology, genetics and biochemistry [74]. Techniques and tools for genetic engineering and fermentation technologies for *S. cerevisiae* have also been extensively developed [74, 76-78], this has aided the genetic engineering *S. cerevisiae* for various purposes. As it occurs widely in nature, *S. cerevisiae* has acquired the ability to tolerate various inhibitors. This ability can be exploited, studied and enhanced to improve the efficiency of *S. cerevisiae* as a biocatalyst in various processes. The ability of *S. cerevisiae* to cope with inhibitory phenolic compounds was of interest in these studies, and in the remaining part of this thesis, the influence of phenolic compounds on *S. cerevisiae*, and the engineering methods used to enhance the natural ability of *S. cerevisiae* to better cope with and metabolize phenolic compounds will be discussed.

CHAPTER 3: Phenolic compounds: Toxicity, stress and response of *Saccharomyces cerevisiae*

3.1 Phenolic compounds

Phenolic compounds have one or more hydroxyl groups attached directly to an aromatic ring [79]. They are a large group of molecules occurring naturally in plants [79, 80]. They are involved in plant growth, development, and defence, and serve as the building blocks of lignin [79, 81]. In addition, they function as signalling molecules, pigments and aromas that can attract or repel insects and offer protection to plants against, fungi, bacteria, and viruses [82]. Phenolic compounds are secondary metabolites in plants [83] and are mostly present as esters or glycosides rather than as free compounds. They also exhibit considerable diversity in structure, ranging from simple molecules such as phenol, vanillin and ferulic acid, to polyphenols such as flavonoids, and polymers such as lignin and tannins [79, 82-84]. The phenolic compounds group comprises of several thousands of compounds, all possessing a core aryl ring to which different functional groups are attached [79], more than 8,000 molecules have been reported in the increasingly growing list of flavonoid family alone [83]. Phenol (Figure 5) is the most basic member of the phenolic group, it is the structure upon which the entire group is based. The aromatic ring in this case is benzene.

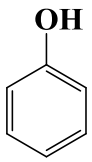

Figure 5: Phenol, the simplest member of the group of phenolic compound.

Small phenolic compounds are biologically active molecules, and are therefore used in various applications in the food, chemical and pharmaceutical industries, often as food preservatives, antioxidant fortifiers and drug molecules [83, 85-90]. Phenolic polymers such as tannins are used commercially as dyes and astringents, and lignin in various industrial applications, commonly as a binder (for example, in the manufacture of ceramics and animal feed pellets), a dispersant (e.g. in cement), an emulsifier (e.g. in pesticides), and as a sequestrant (e.g. in industrial cleaners) [91-94].

3.2 Toxicity of phenolic compounds

While it is beneficial in plant, the antimicrobial activity of phenolic compounds present a significant challenge to bioconversion of lignocellulosic substrates [95]. Although phenolic compounds have been known to be toxic to *S. cerevisiae* [69, 96], the question of difference in toxicity among phenolic compounds towards *S. cerevisiae* was not clearly answered. In this thesis work, the phenolic compounds found in the black liquor, prehydrolysate and oxygen delignification streams supplied by Innventia AB were profiled. Other phenolic compounds commonly found in spruce hydrolysates were compiled from literature and in total, thirteen phenolic compounds were selected based on their persistent presence in spruce derived pulping side streams and hydrolysates (Paper I). A toxicity screening of the thirteen phenolic compounds was done, toxicity limit was defined as the concentration beyond which growth of *S. cerevisiae* was completely inhibited in the presence of the phenolic compound (Paper 1 if the thesis). The first observation was the vast difference in toxicity among the phenolic compounds (Paper I). A typical characteristics observed at this concentration is that the maximum specific growth rate and or the final OD has reduced to about 20% of that of the control cultivation in which there was no phenolic compound. An adaptation of the screening is presented in Table 3. From these results it is clear that the toxicity of phenolic compounds depends on several factors, such as the type and combination of functional side groups present on the compound.

Table 2: The thirteen spruce-derived phenolic compounds screened for their toxicity to *S. cerevisiae* and their toxicity limits

Phenolic Compound	Type	Concentration range tested (mM)	Toxicity limit (mM)
Coniferyl aldehyde	Aldehyde	0.1-2	1.1
Ferulic acid	Acid	0.1- 2.5	1.8
Vanillideneacetone	ketone	0.1- 11	4.2
Homovanillic acid	Acid	0.1 - 11	8.8
Vanillin	Aldehyde	0.1 - 11	9.2
Hydroquinone	Alcohol	0.1 - 11	9.4
Gallic acid	Acid	0.1 - 11	9.4
p-Coumaric acid	Acid	0.1 - 11	9.7
4-Hydroxybenzoic acid	Acid	0.1 - 13	11.6
Homovanillyl alcohol	Alcohol	0.1 - 16	14
Hydroferulic acid	Acid	0.1 - 11	14
Vanillic acid	Acid	0.1 - 16	14.5
Syringic acid	Acid	0.1 - 22	>21

Adapted from paper I.

3.3 Relationship between structure and toxicity of phenolic compounds

From the results of the present work (Paper I) and information obtained from the literature, it could be concluded that the inhibitory influence of phenolic compounds on microbial growth and product yield varies considerably, and is dependent on specific functional groups [59, 69, 97, 98]. However, it is still not clear how these factors combine to make phenolic compounds inhibitory to *S. cerevisiae*. It was, however, observed in this work that the structural features of methoxycinnamaldehydes, in which a combination of a methoxy group, long carbon chain with unsaturated bond and an aldehyde group are together present on an aromatic ring (an example of which is coniferyl aldehyde in Figure 6 below) makes a phenolic compound more inhibitory than a combination of a methoxy group and a long carbon chain with unsaturated bond together with a carboxylic acid on the aromatic ring such as seen in methoxycinnamic acids, a typical example of which is ferulic acid. Structures such as methoxybenzaldehydes (an example of which is vanillin)

that do not have as many unsaturated bonds like in methoxycinnamaldehydes are less inhibitory. Meanwhile, the hydroxyl group seems to contribute the least to toxicity, among the functional side groups on phenolic compounds. Actually, the presence of hydroxyl group on the *ortho*, *meta* and *para* position of an aromatic compounds has been observed to make the aryl ring susceptible to microbial cleavage, thus facilitating the metabolism of such compounds. A typical example of this is the metabolism of catechol and protocatechuate in *S. cerevisiae*, which was facilitated by the position of the hydroxyl groups on the *meta* and *para* carbon atoms on the aryl ring [99]. As discussed in Paper IV, one of the routes through which the conversion of coniferyl aldehyde, ferulic and *p-coumaric* acids has occurred is via guaiacol, subsequent conversion through guaiacol has also been favoured by the location of hydroxyl and methoxy side groups on the aryl rings of the compounds (Paper IV).

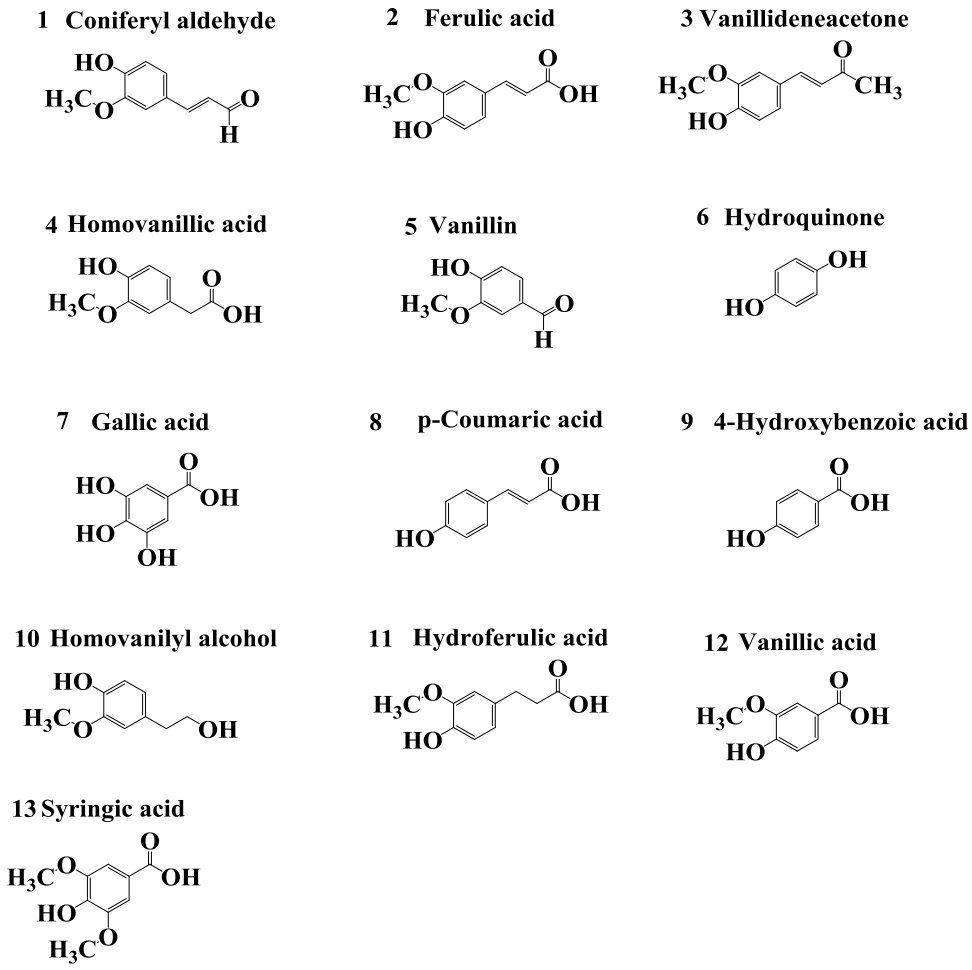

Figure 6: The structures of the 13 phenolic compounds screened for toxicity and showing the different side groups and their locations on the compound.

3.4 Stress and physiological changes elicited in *S. cerevisiae* by the presence of phenolic compounds

Microbial stress can be defined as processes that damage the cell, causing impaired growth or physiological function, or even death unless measures are taken to alleviate it [100, 101]. Stress factors include conditions such as osmosis, pH, temperature, oxidation by reactive oxygen species, nutrient starvation and several other functions that bioactive molecules may induce in a cell [102-105]. The cell is damaged in different ways depending on the chemical and physical properties of the stress factor [106-109]. In the case of phenolic compounds, the mechanisms inducing stress do not appear to be universal, and have not yet been clearly elucidated [110]. Due to the heterogeneity and size of the phenolic compounds, it is difficult to find accurate qualitative and quantitative data to determine the mechanisms of inhibition among these compounds. It has been proposed that phenolic compounds may interfere with the cell membrane of *S. cerevisiae* by influencing its function and changing its protein-to-lipid ratio, as has been demonstrated in bacteria [59, 111]. This suggestion may be supported by the demonstrated ability of polyphenols to adhere to membrane lipids of *S. cerevisiae* [112, 113]. It has also been proposed that phenolic compounds may induce loss of integrity of biological membranes, thereby affecting their ability to be selectively permeable barriers and enzyme matrices [114]. Phenolic compounds such as nonylphenol have been shown to inhibit fungi by uncoupling respiration [115], while phenolic acids have been speculated to cause the destruction of electrochemical gradient by transporting protons back across mitochondrial membranes [114]. Phenolic acids such as benzoic acid are lipophilic and has been reported to tend to accumulate as poorly membrane permeable charged anions, intracellularly in the cell [116]. As a result of the membrane impermeability, the anion is unable to readily diffuse out of the cell [116]. The proton released from the intracellular dissociation of the acid and the intracellular pool of the acid anion is proposed to be a major trigger of stress responses that are elicited in the presence of weak organic acids. Furthermore, H^+-ATPase, War1p and Pdr12p have been reported to be activated by the cells in order to remove the protons and acid anions [116-119]. The process of removal may come at an energy cost to the cells and could partly explain the reduced biomass in the presence of benzoic acid [66, 116].

CHAPTER 4: Phenolic bioconversion and detoxification in *Saccharomyces cerevisiae*

The bioconversion of phenolic compounds is well-known in *S. cerevisiae*. It is also known that glucose is the preferred carbon source for *S. cerevisiae* [120-122]. In the light of this, the question of why *S. cerevisiae* would chose to catabolise and convert phenolic compounds, which are inhibitory and non-preferred carbon sources, in the presence of glucose is interesting. It could be speculated that this is primarily the response of *S. cerevisiae* to a stressor in its environment. In this chapter, the bioconversion of phenolic compounds as a stress response is discussed, based on the evolutionary capability for survival naturally acquired by *S. cerevisiae*.

4.1 Response to phenolic stress in *S. cerevisiae*

The bioconversion of lignocellulosic biomass is a stressful process for the micro-organisms employed in this process due to the unavoidable presence of inhibitory compounds such as phenolic compounds. Stress resistance is therefore a highly desirable phenotype among the microorganisms used in lignocellulosic bioconversion *S. cerevisiae* is confronted with a stressful environment, it will respond to it by attempting to counteract the detrimental effects of the stressor in order to avoid reduced growth disadvantage or even death [123]. *S. cerevisiae* is naturally found in environments such as decaying fruit and fermented plant residues that are rich in ethanol. It is also found naturally in flowers and tree sap. Stress can be induced by the presence of ethanol, and variability in the availability of water, pH, temperature and nutrients, among others [124, 125]. Such natural environments and applied conditions in which *S. cerevisiae* is being used exert various types of stress on the *S. cerevisiae*, pushing the cells to evolve and develop a robust and extensive stress response machinery consisting of various repair and protection mechanisms against different types of stress as it is known in *S. cerevisiae* today. [101, 102, 104, 108, 109, 123, 126-135]. The cellular response of *S. cerevisiae* can often be followed by measuring parameters such as growth, metabolic activity, cell morphology, metabolite abundances, transcript and protein in the cell [136]. While physiological parameters such as growth and cellular morphology can be easily monitored, it is the various molecular mechanisms such as gene regulation, protein synthesis, transcription regulation in the cells that account for the stress response monitored in those parameters

[109, 137, 138]. It has been said that *S. cerevisiae* responds by changing the expression of approximately 1500 genes when exposed to a stressful environment [123]. Of these 1500 genes, about 900 change irrespective of the nature of the stress, and are therefore often referred to as genes of the environmental stress response, and are either repressed or induced [123, 139, 140]. Although the genes that are altered during the exposure of *S. cerevisiae* to phenolic compounds are not completely known, the exposure of *S. cerevisiae* mutants to vanillin, for example, has been reported to result in mutants of up to 76 genes involved in chromatin remodelling, vesicle transport and ergosterol biosynthesis [141]. This suggests that under vanillin stress, cells of *S. cerevisiae* will probably respond by increasing their production of ergosterol, while also upregulating genes to protect and repair chromatin proteins and DNA that are probably damaged by vanillin. Under phenolic stress, apparent signs usually include prolongation of the lag phase, growth inhibition and changes in cell morphology, growth rate, metabolite productivity, substrate consumption and biomass yield [142], these are also shown in Paper I and Paper II of this thesis.

The response of *S. cerevisiae* to stress follows a particular sequence of events. When exposed to a stressor, *S. cerevisiae* initially exhibits a transient change or response that is often epigenetic in nature, and takes place at the transcript level of several genes in the cell [143]. If the stressor is persistent, this will lead to an increase in the rate of mutation and genetic changes that specifically fit the needs of the cell to survive in the environment and overcome the stressor [144, 145]. This process of genetic change or response is more commonly known as adaptive evolution. The response is often manifested phenotypically in the cells. The phenotypic signs are often changes in growth rate, changes in cell morphology or changes in the levels of metabolites produced by the cells. *S. cerevisiae* has been reported to exhibit different physiological changes in the presence of phenolic compounds. For example, phenolic compounds have been shown to reduce the growth rate of *S. cerevisiae* and the yields of ethanol and biomass [69, 146]. In the present work, both increased and reduced production of acetate and glycerol and yields were observed in *S. cerevisiae,* depending on the phenolic compounds involved (Papers I and II). The relationship between the stressor and response in *S. cerevisiae* can be schematically summed up as illustrated in Figure 7 below.

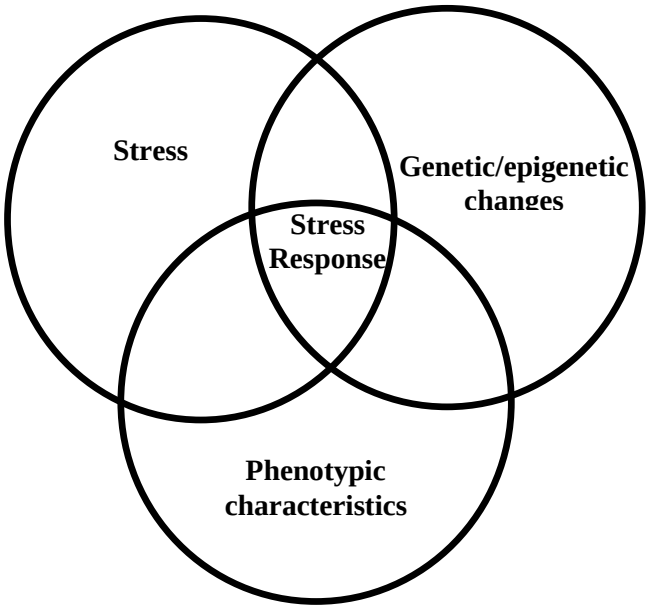

Figure 7: Scheme of environmental and molecular processes behind stress response in *Saccharomyces cerevisiae*.

Apart from the variation in the inhibitory capacity of the 13 phenolic compounds listed in Table 2, the physiological responses of *S. cerevisiae* to these phenolic compounds were also studied (Paper I). Three distinct growth patterns were observed among the 13 phenolic compounds, enabling them to be categorized into three clusters. The first cluster consisted of coniferyl aldehyde (4-hydroxy-3-methoxycinnamaldehyde), homovanillyl alcohol, vanillin, syringic acid and dihydroferulic acid. This cluster caused *S. cerevisiae* to exhibit prolongation of the lag phase as well as a reduction in both the maximum specific growth rate and the final biomass concentration which corresponded to the concentration of phenolic compounds in the medium until a concentration of compound is attained at which a cessation of growth occurred. The second cluster of compounds comprised of p-coumaric acid, hydroquinone, ferulic acid, homovanillic acid and 4-hydroxybenzoic acid while the third cluster was made up of vanillic acid, gallic acid and vanillylidenacetone. The second and third clusters of phenolic compounds had no influence on the lag phase, rather, cluster 2 compounds induced a reduction in the maximum specific growth rate and both cluster 2 and cluster 3 compounds caused a reduction in biomass with increasing concentration of the compounds until the concentration at which growth ceased was reached. An intra-cluster comparison of the phenolic compounds with the physiology of *S. cerevisiae*, using metabolite indicators

such as ethanol, glycerol, biomass and acetate yields, suggested that phenolic compounds belonging to the same cluster have similar inhibitory activity on yeast (Paper I). One of the interesting factors concerning the clusters was that the phenolic compounds making up each cluster were quite diverse in structure and toxicity, although they induced the same physiological influence on *S. cerevisiae*. The question remained, however, as to whether the physiological changes observed in the *S. cerevisiae* in the presence of the phenolic compounds in the different clusters is predicated on similar molecular mechanisms in the cells.

4.2 Catabolism and detoxification of aromatic and phenolic compounds in *S. cerevisiae*

S. cerevisiae has been reported to be able to convert some inhibitory phenolics to less toxic compounds (Paper II). Early attempts have also been made to investigate this conversion, especially the breakage of the aromatic ring, using catechol as a model compound and using several species of yeasts [147]. The conversion of phenolic compounds and the breakage of the aryl ring by *S. cerevisiae* has long been of great interest to scientists because is seen as an essential step in nature's carbon cycle [99]. Coniferyl aldehyde is known to be reduced to coniferyl alcohol and dihydroconiferyl alcohol under fermentative conditions [69] while it is converted to cinnamic acids under aerobic conditions (Paper II). Ferulic acid and other cinnamic acids have also been reported to be catabolised by *S. cerevisiae* [148-150]. The bioconversion of phenolic compounds in the presence of glucose was also observed in this work (Paper 2), although phenolic compounds are not the preferred carbon source for *S. cerevisiae*. It is therefore debatable whether the bioconversion of the otherwise toxic phenolic compounds is a response to the stress on the cells resulting from these compounds. The cells are challenged by a stressor, and respond by altering their gene regulation, such that enzymes promoting the survival of the cells in the presence of the phenolic compounds are increasingly produced. These enzymes enable the cells to catabolize the stressor when possible, and make the environment more conducive to cell growth. It is thus plausible to interpret the bioconversion process of phenolic compounds in the presence of glucose as a survival strategy, rather than a nutritional preference of the cell. It has been suggested that the conversion of phenolic compounds takes place via the β-ketoadipate pathway, which is common in many microorganisms including *S. cerevisiae* [99, 151-153]. The β-

ketoadipate pathway is employed by microorganisms to degrade aromatic compound via ortho-cleavage. While one branch converts protocatechuate, derived from phenolic compounds, to beta-ketoadipate, while the other branch converts catechol, generated from various aromatic hydrocarbons, amino aromatics, and lignin monomers, also to beta-ketoadipate which is then converted to tricarboxylic acid cycle intermediates [151]. This gives microorganisms a two way option to metabolise otherwise complex and recalcitrant aromatic compounds.

While *S. cerevisiae* cannot degrade the aryl ring in certain aromatic compounds such as benzoic acid, it can degrade catechol [99, 154]. In Paper IV, in which the conversion of coniferyl aldehyde, ferulic acid and p-coumaric acid is reported it was evident from the conversion products that *S. cerevisiae* converts coniferyl aldehyde, ferulic acid and *p*-coumaric acid into guaiacol via several intermediates. It was thus hypothesized that guaiacol is converted into catechol via hydrolysis of the methoxy group on the *ortho* carbon atom of guaiacol. The catechol is then converted through the β-ketoadipate pathway. This presents at least one catabolic route through which coniferyl aldehyde, ferulic acid and *p*-coumaric acids are converted. Several phenolic intermediates that are formed during the catabolism of the three phenolic compounds, are also converted by *S. cerevisiae* via the same route. This strongly indicates therefore that this conversion route in *S. cerevisiae* is valid for the conversion of several other phenolic compounds. The bioconversion of phenolic compounds, either to less toxic derivatives or complete degradation through the breakdown of the aryl ring, can thus be described as a detoxification response in the cells that have survived the stress of the compounds, by inducing the genes needed for survival and producing the enzymes required for bioconversion.

4.3 Enzymes, genes and pathways for phenolic catabolism in *S. cerevisiae*

A stepwise conversion process involving several enzymatic steps was observed in the conversion of coniferyl aldehyde, ferulic acid and *p*-coumaric acid (Papers II and III). For instance, the conversion of coniferyl aldehyde to ferulic and other cinnamic acids is an oxidation reaction. In the cell, in the presence of oxygen an oxidoreductase is required to catalyse such a reaction. Later in the conversion of the products from coniferyl aldehyde, ferulic acid and p-coumaric acid, other phenolic acids and phenolic alcohols are formed.

A fascinating observation in the conversion of coniferyl aldehyde, ferulic acid and *p*-coumaric acid is that the conversion products were very similar for all three phenolic compounds. As presented in Paper II and Paper IV, and illustrated in Figure 8, coniferyl aldehyde was converted to phenolic acids, including ferulic and *p*-coumaric acids, which were then subsequently converted to phenolic alcohols. This suggests that the conversion routes for these three compounds is the same in *S. cerevisiae*, and that conversion involves the activity of several enzymes, some of which are proposed in the scheme below.

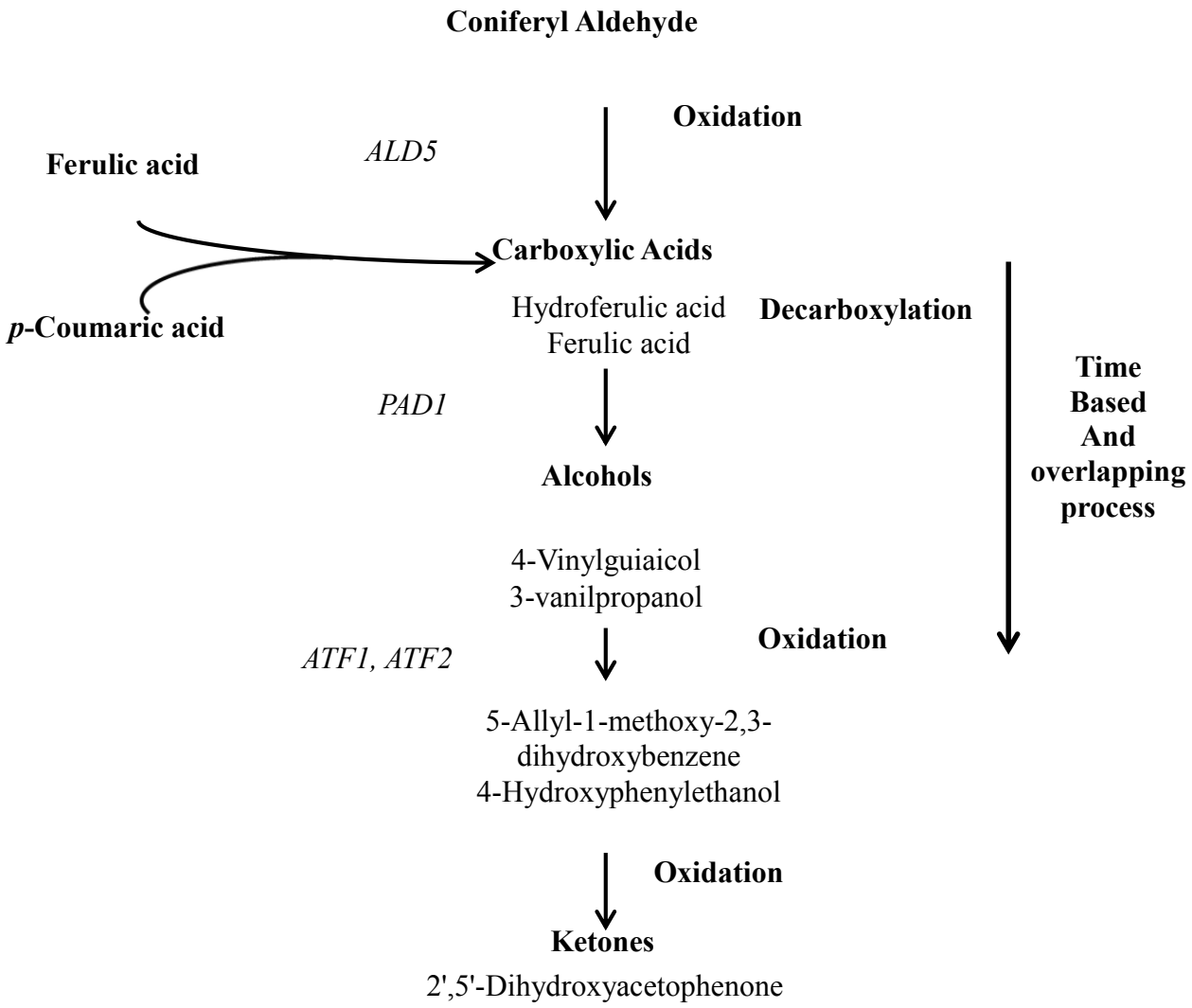

Figure 8: Proposed conversion pathway for phenolic compounds using the conversion of coniferyl aldehyde, ferulic acid and p-coumaric acid as template compounds. (From Paper IV)

Another observation made regarding the conversion of coniferyl aldehyde, ferulic acid and *p*-coumaric acid is that the conversion products are not formed at the same time, but in a sequential manner, which suggests the conversion of one intermediate compound into another. As illustrated in Figure 9, a conversion sequence starts with the conversion of the alkanal group into a carboxylic acid group. This is followed by decarboxylation and a series of oxidation steps that yield successively less toxic compounds. This conversion sequence further supports the suggestion that several enzymes play different roles in the conversion process.

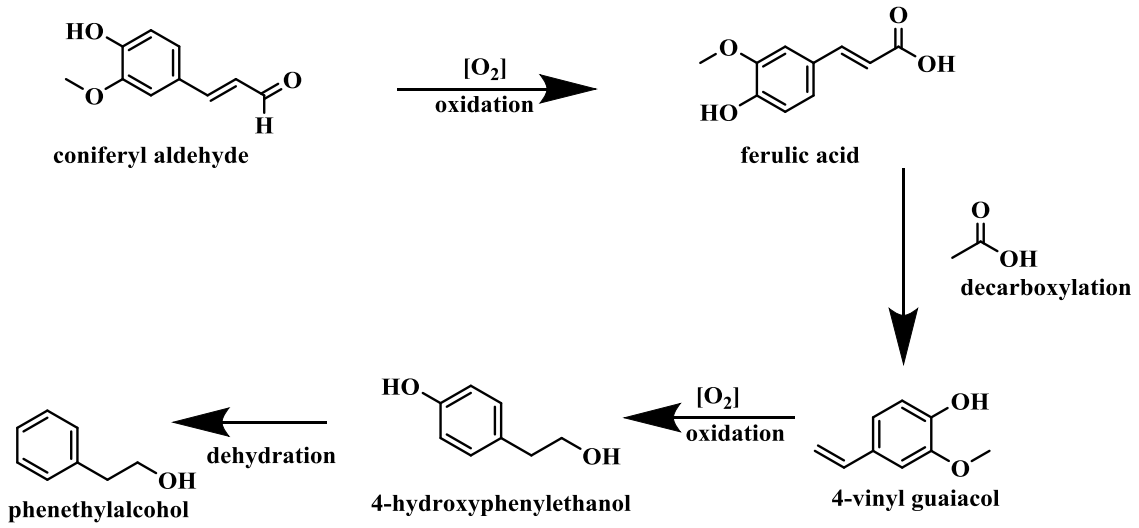

Figure 9: A simplified conversion sequence for coniferyl aldehyde by *S. cerevisiae.* (From Paper II)

It has been confirmed that various enzymes are involved in the conversion of phenolic compounds by *S. cerevisiae*. Phenylacrylic acid decarboxylase (Pad1) which confers resistance to cinnamic acids in *S. cerevisiae S. cerevisiae*, is known to be involved in the conversion of cinnamic acid via decarboxylation [155, 156]. Also, Fdc1p, a ferulic acid decarboxylase, has also been reported to be essential for the decarboxylation of phenylacrylic acids in *S. cerevisiae* [157, 158]. Many more enzymes are expected to be involved in the catabolism of phenolic compounds in *S. cerevisiae*, either by directly catalysing a single step or a series of steps in the conversion, or by signalling other proteins that are directly involved in the conversion of phenolic compounds. It has been reported that. Yap1p, Atr1p and Flr1p are reported to be involved in conferring resistance to phenolic compounds [159], Yap1 is a transcription factor that reduces oxidative stress, and is involved in general stress response, while both Atr1p and Flr1p act as efflux pumps

against drugs and toxins that enter the cell [160-164]. Thus, while proteins such as cytochrome p450 are known to exhibit peroxidase activity in *S. cerevisiae* by oxidation of guaiacol [165], or Pad1p and Fdc1p perform decarboxylation, the roles of other proteins such as Yap1, Atr1p and Flr1p have been less direct in conferring resistance on *S. cerevisiae* against phenolic compounds. Yap1p, a member of the AP-1 family of transcription factors is involved in oxidative stress response by activating the transcription of anti-oxidant genes as a response to oxidative stress [166]. It is also known to be involved in resistance to hydrogen peroxide and compounds that alter the redox status in the cell [160, 167]. Yap1p has been reported to regulate several anti-oxidant genes including *TRX2, TRR1, GLR1* and *GSH* [129, 168, 169]. Therefore, while yap1, Atr1p and Flr1p have not been directly reported to be involved in conversion of phenolic compounds, their involvement in conferring resistance to *S. cerevisiae* against phenolic compounds via activation of other genes (done by Yap1p) to ease oxidative stress induced by the phenolic compounds or by facilitating the removal of the compounds from inside the cells as characteristic of Atr1p and Flr1p, qualifies them to be recognized as belonging to a group of proteins involved in phenolic resistance and catabolism in *S. cerevisiae*. In all, evidences point to the fact that *S. cerevisiae* use a combination of several enzymes, proteins and genes to catabolise phenolic compounds.

4.4 Products of phenolic catabolism in *S. cerevisiae*

The catabolism of phenolic compounds by *S. cerevisiae* has long been of interest, and extensive efforts have been devoted to metabolically engineering *S. cerevisiae* to obtain strains with the ability to produce specific phenolic compounds such as resveratrol and *p*-coumaric acid [158, 170, 171]. As reported in Papers II to IV, the conversion of certain phenolic compounds such as coniferyl aldehyde, ferulic acid and *p*-coumaric acid by *S. cerevisiae* yields several products. Since the catabolic process is a series of cleaving steps and steps that alter the nature of the side groups decorating the compounds, the intermediates are mostly phenolic compounds, and the aromatic rings are retained until very late in the conversion process, when they are also cleaved. The product profiles of the conversion reveal several intermediate compounds that have pharmaceutical, nutritional and chemical importance (Paper II). Thus, the capability of *S. cerevisiae* to produce these compounds of interest can be enhanced and exploited for industrial purposes by producing value-added chemicals from lignocellulosic side streams that are

rich in phenolics but poor in fermentable sugars, for example, prehydrolysate, black liquor and the oxygen delignification side stream. While the phenylpropanoid pathway is clearly understood in plants, the bioconversion of phenolic compounds is still a grey area in *S. cerevisiae*. A route for the catabolization of specific phenolic compounds such as coniferyl aldehyde, ferulic acid and *p*-coumaric acid by *S. cerevisiae* was suggested in Paper II, but this remains to be confirmed.

CHAPTER 5: Improvement of phenolic tolerance in *Saccharomyces cerevisiae* by metabolic engineering.

Metabolic engineering involves the application of recombinant DNA methodologies to alter the genetic and regulatory functions within cells in order to confer new traits on them or to optimize the production of metabolites of interest [172-174]. They can also be used to incorporate biochemical pathways or components of existing pathways in one organism into another where it is lacking [173]. The metabolic activities of cells are employed in a large variety of processes, ranging from the production of chemicals and pharmaceuticals, to waste treatment, and various processes in the food industry. As the tools for metabolic engineering gets better, and biological and biochemical processes in cells are better understood, the use of metabolic engineering as a tool for conferring new traits on different species of organisms has increased [74]. The metabolism of the native organism is often not optimal for its application. Therefore, the primary aim of metabolic engineering is to develop new strains of organisms that meet defined requirements for specific production processes, either to develop tolerance against stress inducing elements in a production process or facilitate the production of valuable microbial products on a profitable and sustainable scale in a cost effective manner [173, 175-177].

The goal of metabolic engineering in medicine or biotechnology is often to obtain a high yield of the specific metabolites produced by the engineered organism, a typical example of which is the industrial production of L-amino acids for various purposes [178, 179]. Although chemical synthesis still dominates production in the chemical industry, metabolic engineering has a significant advantage over synthetic organic chemistry as it employs biological mechanisms in living systems for the production of natural products such as active pharmaceutical ingredients, many of which are still too complex to be chemically synthesized, yet highly sought after [173, 180].

Various strains of yeasts, including *S. cerevisiae*, as well as different strains of bacteria, have been engineered to produce or metabolize phenolic compounds of interest such as eugenol and *p*-coumaric acid [158, 181-183]. Although there are many benefits of metabolic engineering, and the fermentation of substrates such as lignocellulosic substrates by microorganisms represents an attractive route for the manufacture of

industrial chemicals from renewable resources, the approach is not without problems as product yields are not always as high as desired [184]. Metabolic engineering approaches have been used to develop strains of microorganisms that can utilize components of lignocellulosic substrates that are not naturally utilized by such microorganisms; typical examples are the metabolic engineering of *S. cerevisiae* for the utilization of xylan and xylose [185-187]. Another area in which metabolic engineering has been useful is in conferring tolerance on microorganisms [74, 188]. The use of metabolic engineering to confer tolerance on microorganisms is discussed in this chapter, specifically strategies that increase the tolerance of *S. cerevisiae* strains to higher concentrations of the phenolic compounds studied in this work. Increasing catabolism has the potential not to only provide strains with increased tolerance to inhibitors, but also to produce conversion products that are of interest for various biotechnological applications.

5.1 Metabolic engineering as a tool for conferring tolerance to inhibitors

It is sometimes necessary to induce or improve the tolerance of microorganisms in biotechnological applications. For instance, in the case of fermentative microorganisms used for the bioconversion of lignocellulosic substrates, the microorganism of interest should be tolerant to the inhibitors present in the substrates. Metabolic engineering has therefore been a vital tool in conferring tolerance against inhibitors on microorganisms [189, 190]. The effects of many lignocellulosic inhibitors such as acetic acid, formic acid, furfural, HMF and several phenolic compounds on *S. cerevisiae* have been individually and comprehensively studied at molecular levels using various omics tools and cell physiology [70, 102, 109, 159, 190-192]. With the knowledge, resistance to such inhibitors have been selectively attempted and often achieved through metabolic engineering approaches for the development of strains [190, 193-195]. Metabolic engineering is therefore a useful platform for the development of tolerance in microorganisms.

5.2 Metabolic engineering approaches to developing phenolic resistance or catabolism in *S. cerevisiae*

At the beginning of these studies, it was hypothesized that there was a relationship between the catabolism of phenolic compounds and tolerance, in other words, that

increased catabolism of phenolic compounds by *S. cerevisiae* also confers increased tolerance to phenolic compounds. Two strategies for the development of *S. cerevisiae* strains with increased catabolism of phenolic compounds were employed to test this hypothesis. These strategies are discussed below, together with other metabolic engineering strategies reported in the literature.

Since bioprocesses often require that cells undergo genetic modifications, which alter the original metabolic balance in the cell, manipulation of the metabolism of cells must be done in a way that increases the chances of obtaining a feasible, and eventually a superior, bioprocess. Several strategies are used in metabolic engineering of *S. cerevisiae* against phenolic compounds, the approach chosen in specific cases depend mostly on the eventual application of the strain. Some of the most common strategies in metabolic engineering are as below and a summary is presented in table 3.

5.2.1 Heterologous expression of genes of interest

The heterologous expression of genes of interest present from other organisms in the *S. cerevisiae* has been used to develop desirable traits in *S. cerevisiae.* As a typical example of application of heterologous expression in developing increased tolerance in *S. cerevisiae* against phenolic compounds, laccase has been heterologously expressed in *S. cerevisiae* leading to an improved resistance of *S. cerevisiae* against phenolic compounds [195]. Laccase, is a copper enzyme common in nature and among several species of fungi, it causes oxidative polymerization of phenolic compounds such as hydroquinones and catechol [196, 197].

The first strategy used in this thesis work, to develop a *S. cerevisiae* strain named *B_CALD* was the heterologous expression of a coniferyl aldehyde dehydrogenase *CALDH* from *Pseudomonas sp* HR199, this was described in Paper III. *B_CALD* was engineered for improved catabolism of coniferyl aldehyde, one of the phenolic compounds of interest to the thesis. *CALDH* gene codes for the similarly named Caldh enzyme which is known to facilitate the conversion of coniferyl aldehyde in the host *Pseudomonas*. The expression of *CALDH* in *S. cerevisiae* enhanced the specific conversion of coniferyl aldehyde up to 27 times in comparison to the control strain. Heterologous expression of enzymes has also been used to express the phenyl propaniod

pathway in *S. cerevisiae*, many phenolic compounds of interest have been synthesised in *S. cerevisiae* as a result of this [158, 171, 198-200].

5.2.2 Overexpression of genes encoding relevant enzymes of a relevant biosynthetic pathway

The overexpression of genes encoding relevant enzymes relevant in a particular biosynthetic pathway that are native to *S. cerevisiae* is another strategy employed in the development of *S. cerevisiae* strains with increased tolerance to and bioconversion of phenolic compounds. *PAD1* and *FDC1* have both been known to code for enzymes that facilitate the decarboxylation of cinnamic acids, and overexpression of these genes have been shown to improve the resistance of *S. cerevisiae* to phenolic compounds [156, 201].

The second approach that was used in developing a *S. cerevisiae* strain named *APT_1*, with increased catabolism and resistance to phenolic compounds is the overexpression of *ALD5, PAD1, ATF1* and *ATF2*, all native to *S. cerevisiae*, I have reported this approach in paper IV which is focused on the aspect of my thesis work in which I have worked on developing a *S. cerevisiae* strain with increased catabolism of coniferyl aldehyde, ferulic and *p*-coumaric acids. I hypothesised the enzymes Ald5, Pad1, Atf1 and Atf2 from the four genes are involved in the conversion of phenolic compounds in *S. cerevisiae*. I have hypothesised that Ald5 is the oxidoreductase involved in the conversion of coniferyl aldehyde to cinnamic acids, Pad1 is responsible for the conversion of the cinnamic acids via decarboxylation to phenolic alcohols while the Atf1 and Atf2 are responsible for the formation of various other phenolic alcohols from the immediate products of the decarboxylation of the cinnamic acids.

5.2.3 Deletion of genes vital to competing metabolic pathways

The deletion of genes vital to competing metabolic pathways is another strategy that has been used to facilitate the catabolism of phenolic compounds. This has been demonstrated in the production of specific phenolic metabolites via biotransformation of other phenolic compounds in *S. cerevisiae*. For example, in the production of resveratrol from *p*-coumaric acid or the bioconversion of aromatic amino acids into *p*-coumaric acid, genes that could either facilitate further conversion of the product of interest or divert intermediate products into undesirable side products were deleted [158, 171].

5.2.4 Expression of genes that serve other functions that is associated with is associated with the biochemical process of interest in the cells

The expression of genes that are not directly involved with the biochemical process of interest but are involved in other processes that influence the biochemical process of interest, is a strategy that has been used extensively in metabolic engineering. For instance, spermidine have been reported to possess different functions in the protection of DNA from reactive oxygen species [202], overexpression of spermidine synthase, *SPE3* was shown to increase the tolerance of *S. cerevisiae* in corn stover hydrolysate that was not said to have been detoxified [189]. Since corn contains several phenolic compounds alongside other inhibitors, overexpression of *SPE3* therefore can be said to increase the tolerance of *S. cerevisiae* against phenolic compounds and other lignocellulosic inhibitors [189].

5.2.5 Enzyme engineering

Enzyme engineering is another strategy that has long been in use in metabolic engineering. Although this strategy has been very rarely demonstrated in developing *S. cerevisiae* strains with increased tolerance to phenolic compounds, it holds great potential in developing *S. cerevisiae* strains with enzymes that are highly specific for certain phenolic substrates and can more effectively convert them. The closest approach to rational enzyme engineering has been directed molecular evolution. In a very successful example, the enzyme Cytochrome c peroxidase, a peroxidases that aid the conversion of guaiacol in *S. cerevisiae* has been engineered with the resulting mutants possessing a 300-fold increased activity against guaiacol and an up to 1000-fold increased specificity for guaiacol, relative to that for the natural substrate [165].

Application of the strategies described above has resulted in a number of strains of *S. cerevisiae* with increased bioconversion or tolerance to phenolic compounds. With each successful case of heterologous expression of an enzyme, part of a pathway, or overexpression of an endogenous gene, the success of the strategy has been closely associated with the availability of detailed knowledge. Furthermore, each successful result involved engineering directed towards a specific phenolic compound, such as guaiacol, or a small group of phenolic compounds sharing similar structures, such as the

cinnamic acids. Although successful attempts to engineer *S. cerevisiae* with increased tolerance to phenolic compounds have been reported, the approach is not without problems. This could explain the relatively small number of research articles reporting success in this field. The development of tolerance to phenolic compounds face some significant challenges; one is the limited understanding of specific cellular target of phenolic compounds in *S. cerevisiae*, corresponding cellular responses and molecular processes that are triggered in *S. cerevisiae* when confronted with phenolic compounds. It is not certain whether phenolic targets and cellular response are similar among many phenolic compounds, this is mainly due to the diversity among the large family of phenolic compounds. Another challenge is that the attending consequences of metabolically engineering cells with new functions or enzymes is not usually known ahead of an engineering strategy. A well designed metabolic engineering strategy may not yield the desired result because the whole cellular machinery has to adjust for and cope with the newly introduced metabolic process. Several consequences such as slower growth, reduced biomass yield, accumulation of undesired metabolite, to mention a few of such phenomenon that are usually placed under the umbrella of "metabolic burden", often result from metabolic engineering. In the first strategy used in this thesis (Paper III), despite the significant increase in specific conversion of coniferyl aldehyde by the engineered strain *B_CALD*, a cessation of growth during the coniferyl aldehyde conversion phase in the strain was observed. It was speculated that this may have been due to the demand for NAD^+ required by the enzyme to convert coniferyl aldehyde. The expression of *CALDH* altered the physiology of *S. cerevisiae*.

Table 3: Some demonstrated metabolic engineering strategies for developing *S. cerevisiae* strains with improved tolerance and metabolism of phenolic compounds.

	Principle	Strategy	Benefits	Limitations
1	Heterologous expression of genes of interest	Expression of laccase [195] Construction of phenylpropanoid pathway [158, 171, 198-200] Expression of *CALDH* (Paper III).	Increased conversion of phenolic compounds achieved (Paper III) [195] Phenolic metabolite of interest produced [158, 171, 198-200]	Prolonged lag phase (Paper III).
2	Overexpression of genes encoding relevant enzymes of a relevant biosynthetic pathway	Expression of *PAD1, PDC1* and other genes coding for enzymes of interest [156, 201], Paper IV.	Increased decarboxylation and conversion of cinnamic acids. Better physiological performance in the presence of cinnamic acids	Metabolic burden.
3	Deletion of genes vital to competing metabolic pathways	Disruption of target genes [158, 171]	Some accumulation of phenolic metabolites of interest was achieved.	Low tolerance to substrates. Lower than desired productivity of metabolite of interest.
4	Expression of genes that serve other functions that is associated with is associated with the biochemical process of interest in the cells	Overexpression of *SPE3* and disruption of *OAZ1 ODC* and *TPO1* to accumulate spermidine [189]	Enhanced tolerance of *S. cerevisiae* against major fermentation inhibitors achieved	Metabolic burden. Prolonged lag-phase
5	Enzyme engineering	Targeted mutagenesis substrate [165]	Increased specificity for and conversion of guaiacol.	Screening a large number of clones.

CHAPTER 5: Improvement of phenolic tolerance in *Saccharomyces cerevisiae* by metabolic engineering.

CHAPTER 6: Conclusions

The challenges of converting lignocellulosic substrates into fuels and chemicals are numerous, and tackling the effects of phenolic inhibitors on the classical fermenting organism *S. cerevisiae* is one of the challenges that remains to be solved. Furthermore, the use of phenolic-rich substrates for the production of specific phenolic metabolites will only be a viable prospect provided we have an adequate understanding of the catabolism of phenolic compounds by *S. cerevisiae*. The work described in this thesis was therefore focused on the study of the metabolism of phenolics in *S. cerevisiae*.

The complexity associated with phenolic compounds arises mainly from the diversity of the compounds in the phenolic family. Each compound is unique and may have its own influence on *S. cerevisiae*. The functional groups attached to each compound are responsible for the uniqueness of each phenolic compound, and determine the toxicity of the phenolic compounds. From the results of the studies presented in this thesis it could be concluded that the nature, a combination of the functional side groups and the saturation of the bonds on the phenolic compounds determine how toxic the compounds are. This is responsible for the significantly higher toxicity in coniferyl aldehyde that possess a methoxy group, an hydroxyl group and an aldehyde group anchored via a longer alkyl chain with unsaturated bond compared to vanillin, which also possess a methoxy group, an hydroxyl group and an aldehyde group but on a short, saturated anchorage.

The mechanisms of action also seem to vary among the phenolic compounds, and our knowledge concerning the mechanisms of action of individual phenolic compounds and their effects on *S. cerevisiae* is still limited. However, the physiological effects of some of the phenolic compounds are similar. In the study reported in Paper I, the physiological response of *S. cerevisiae* to thirteen phenolic compounds with regard to their toxicity helped to distinguish three clusters of compounds. However, it still remains to be elucidated whether the physiological changes observed in *S. cerevisiae* that determined the clustering of the compounds were the result of a common molecular influence of the compounds in each cluster on *S. cerevisiae*, or not.

An important but often overlooked aspect is the ability of *S. cerevisiae* to initiate survival mechanisms when challenged by a hostile environment. One such mechanism is the transformation of inhibitors; in this work, phenolic compounds. In the study on coniferyl aldehyde, ferulic acid and *p*-coumaric acid (Paper II), the conversion of the three phenolic compounds by *S. cerevisiae* was observed. The conversion of phenolic compounds by *S. cerevisiae* is an interesting phenomenon that has also been reported previously in the literature. Surprisingly, coniferyl aldehyde, which is the most inhibitory compound, was the most rapidly converted, and the highest number of conversion products was derived from coniferyl aldehyde Furthermore, ferulic acid and *p*-coumaric acid share a conversion product profile with coniferyl aldehyde. The rate of conversion varies among the compounds, but similarities in the conversion product profiles led to the conclusion that the conversion route for these three phenolic compounds is the same, as was reported in Paper II.

The ability of *S. cerevisiae* to catabolize phenolic compounds can be exploited to improve our understanding of the chemical processes taking place in the cells in order to develop strains of *S. cerevisiae* that are not only tolerant to phenolic compounds, but can produce specific phenolic metabolites through catabolism. The chemistry of the reactions taking place in the cells can also provide valuable insight into the enzymes and genes involved in the catabolic process, providing valuable information on which genes and enzymes should be targeted in metabolic engineering. The diversity of phenolic compounds prevents the use of an all-encompassing strategy in engineering phenolic metabolism or tolerance in *S. cerevisiae*. However, strains can be developed by engineering several genes and enzymes simultaneously. A combinatorial strategy in which all the enzymes involved in the bioconversion of a small group of compounds are targeted appears to be more promising, as evident from the results presented in Paper IV.

Two strategies were employed in an attempt to improve the catabolism of phenolic compounds by *S. cerevisiae*. The first involved the heterologous expression of an enzyme known to catabolize phenolic compounds (*CALDH* in the *B_CALD* strain) and the other, multiple overexpression of four endogenous genes hypothesized to play active roles in the catabolism of coniferyl aldehyde, ferulic acid and *p*-coumaric acid (*ALD5*, *PAD1*, *ATF1* and *ATF2* in the strain *APT_1*). Although the heterologous expression of *CALDH* did not lead to increased tolerance, and a temporary cessation of growth was observed

during the conversion of coniferyl aldehyde, both strategies yielded strains of *S. cerevisiae* with increased catabolism of coniferyl aldehyde, ferulic acid and *p*-coumaric acid. Improving the catabolism of phenolic compounds and developing strains that can utilize substrates rich in phenolic compounds more efficiently, could thus be effective approaches in developing phenolic-tolerant strains of *S. cerevisiae.*

CHAPTER 7: Future perspectives

This thesis describes the studied carried out to obtain new knowledge and improve our understanding of how *S. cerevisiae* catabolizes phenolic compounds. Efforts have also been devoted to developing new strains of *S. cerevisiae* with improved catabolism of, and tolerance to, phenolic compounds. While significant challenges remain, I am optimistic that the small steps taken in this work have moved us closer to the development of a "super yeast" with all the safety characteristics required for the industrial production of second generation fuels and chemicals using lignocellulosic biomass as the raw material. In particular, I believe that the work contained in this thesis has taken us a little farther in the development and use of *S. cerevisiae* as a biocatalyst for production of chemicals from lignin residues, this is of currently of significant interest. This work is also complimentary to the knowledge pool available on the tolerance of *S. cerevisiae* to organic acids and furaldehydes and utilization of C5 sugars.

The catabolism of phenolic compounds by *S. cerevisiae* is of considerable interest and relevance in several industries and applications. However, several challenges remain to be overcome before a strain of *S. cerevisiae* can be developed which can catabolize phenolic compounds to at the concentrations necessary in commercial applications. It is also important to be able to direct the catabolic process towards specific products of interest. The immediate challenges are the development of a more robust *S. cerevisiae* strain and the manipulation of the catabolic process in *S. cerevisiae* to achieve the desired products. In order to develop a strain of *S. cerevisiae* that is more tolerant to phenolic compounds, more detailed studies must be carried out on cellular targets in *S. cerevisiae*. Also, the mechanism behind the tolerance of *S. cerevisiae*, or at least the most important genes and cellular processes involved in its resistance, to phenolic compounds must be elucidated in order to develop a *S. cerevisiae* strain that is more robust to phenolics inhibition. Bearing in mind the fact that the mechanisms governing inhibition probably vary between different phenolic compounds, this is a serious challenge.

Although a catabolic route for phenolic compounds has been proposed in this thesis, it is evident that other enzymes may be involved in the conversion of phenolics. In order to

be able to engineer the catabolism of phenolic compounds by *S. cerevisiae* effectively, the entire catabolic route must be elucidated, and the products of the catabolism of each phenolic compound must also be known. Once this has been achieved, strains of *S. cerevisiae* can then be tailored for the production of specific catabolites from substrates containing specific phenolic compounds. Regulation of the catabolic process is vital in developing strains incorporating mechanisms that allow the catabolism of specific phenolic compounds to be controlled, including termination at the desired catabolite. This emphasizes the need to understand steps in the catabolic process that are not yet clearly understood. In our quest to understand the catabolic process in detail, all the enzymes involved in the conversion process must be identified. Since the metabolism of *S. cerevisiae* is not optimized for the catabolism of phenolic compounds, it will have to be metabolically engineered to improve the conversion of phenolics. This could mean overexpressing or deleting genes for specific enzymes, targeted mutagenesis or using different types of promoter genes to induce the expression of the enzymes. Many of the intermediate compounds are of interest, but due to their transient nature, they are soon converted into other compounds. In order to be able to extract these compounds, the conversion pathway must be further engineered so that conversion ceases when the phenolic metabolite of interest has been reached.

Understanding the metabolism of phenolic compounds in *S. cerevisiae* is crucial to bioeconomy. The concept of a biorefinery in which every component of the feedstock is maximally utilized is enhanced when feedstocks such as soda pulping side streams, which are rich in aromatic lignin residues and low in fermentable sugars, can be utilized for the production of chemicals. Furthermore, the range of products that could be derived from the conversion of one phenolic compound into another has been demonstrated in this work, by studying the conversion of coniferyl aldehyde, ferulic acid and *p*-coumaric acid. A wide range of products are already being chemically synthesised from lignin on an industrial scale, however a large amount of lignin is still being combusted for energy production. There is thus a room and a need to expand the application of lignin, especially in the biochemical industry.

Plants synthesize a broad range of secondary metabolites, including phenolics, which constitute a natural source of diverse bioactive molecules of relevance in the food, chemical and pharmaceutical industries. The bulk of secondary metabolites from plants

are still not being utilized effectively due to the cost of processing. The chemical synthesis of many of these plant-derived high-value chemicals, such as phenolics used as flavouring and colourants in foods, and as cosmetic and pharmaceutical additives, is often expensive and not environmentally friendly. I hope that the work presented in this thesis has provided a basis for the microbial production of such high-value phenolics in fermentative bioprocesses that are scalable and sustainable, ultimately serving as alternative production platforms to commercial chemical synthesis. This would add value to forest-based industries where waste production and management is a constant challenge. Approximately 20-100 litres of waste water is produced for every ton of wood processed. This waste water is generally low in fermentable sugars but rich in tannin, derivatives of lignin and other chemicals. The development of strains of *S. cerevisiae* that can efficiently transform the phenolic residues in such waste water will bring us closer to the realization of an integrated biorefinery, in which all the components of lignocellulosic biomass are converted into value-added products and waste is reduced.

I hope the work presented in this thesis has increased awareness of the opportunities offered by the bioconversion of aromatic residues into specific high value chemicals from depolymerized lignin, using metabolically engineered strains of *S. cerevisiae*.

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Paper I

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The chemical nature of phenolic compounds determines their toxicity and induces distinct physiological responses in *Saccharomyces cerevisiae* in lignocellulose hydrolysates

Peter Temitope Adeboye, Maurizio Bettiga and Lisbeth Olsson*

Abstract

We investigated the severity of the inhibitory effects of 13 phenolic compounds usually found in spruce hydrolysates (4-hydroxy-3-methoxycinnamaldehyde, homovanilyl alcohol, vanillin, syringic acid, vanillic acid, gallic acid, dihydroferulic acid, *p*-coumaric acid, hydroquinone, ferulic acid, homovanillic acid, 4-hydroxybenzoic acid and vanillylidenacetone). The effects of the selected compounds on cell growth, biomass yield and ethanol yield were studied and the toxic concentration threshold was defined for each compound. Using Ethanol Red, the popular industrial strain of *Saccharomyces cerevisiae*, we found the most toxic compound to be 4-hydroxy-3-methoxycinnamaldehyde which inhibited growth at a concentration of 1.8 mM. We also observed that toxicity did not generally follow a trend based on the aldehyde, acid, ketone or alcohol classification of phenolic compounds, but rather that other structural properties such as additional functional groups attached to the compound may determine its toxicity. Three distinctive growth patterns that effectively clustered all the compounds involved in the screening into three categories. We suggest that the compounds have different cellular targets, and that. We suggest that the compounds have different cellular targets and inhibitory mechanisms in the cells, also compounds who share similar pattern on cell growth may have similar inhibitory effect and mechanisms of inhibition.

Keywords: Phenolics; Toxicity; Inhibition; Tolerance; Conversion; *Saccharomyces cerevisiae*

Introduction

Lignocellulose, primarily made up of carbohydrates and lignin, has been billed as the most abundant material on earth (Chandel *et al.* 2011). Next to carbohydrates, aromatic compounds are the second most abundant class of organic compounds in nature (Boll *et al.* 2002). It has been claimed that aromatic compounds, including phenolics make up about 25% of the earth's biomass (Gibson and Harwood 2002). This abundance is significant to the usage of plants and plant residues as important resources in second generation biofuel and chemicals production.

Phenolic compounds are secondary metabolites that are synthesized by plants via the pentose phosphate, shikimate and phenylpropanoid pathways (Randhir *et al.* 2004). They form the building blocks of lignin and they play crucial role in plants resistance to diseases and infections (Hutzler *et al.* 1998, Nicholson and Hammerschmidt 1992, Vance *et al.* 1980, Vanholme *et al.* 2010). Lignin in itself is a natural polymer that is primarily made up of phenylpropane units derived from guaiacol, p-hydroxyphenol and syringol, all interconnected in a C-C bond (Dorrestijn *et al.* 2000, Mcdonough 1983, Nenkova et al. 2011). Phenolic compounds are directly involved in various plant physiological processes and plant defense mechanisms against microbial infections (Bhattacharya *et al.* 2010, Blum *et al.* 1999, Bravo 1998, Hutzler *et al.* 1998, Muller *et al.* 1964). In addition, their antimicrobial, antioxidant activity, and their various other dietary and pharmaceutical properties make them highly relevant to food and pharmaceutical industries (Balasundram *et al.* 2006, Benavente-Garcia *et al.* 1997, Hertog *et al.* 1993, Puupponen-Pimia *et al.* 2001, Scalbert and Mazur 2002).

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On the other hand, the beneficial effect of the antimicrobial activities of phenolic compounds which is beneficial to plants present a significant challenge to the production of second generation bioethanol and other chemicals from plant residues and lignocellulosic materials (Klinke *et al.* 2004). During biofuel production, plant biomasses are first subjected to pre-treatment processes and hydrolysis in order to breakdown their structures and adapt them to forms accessible by enzymes for fermentation and bioconversion. Diverse phenolic compounds are formed as residues of lignin degradation during these wood and plant residue pre-treatment processes for hydrolysate production and wood pulping (Guss 1945, Klinke *et al.* 2004, Larsson *et al.* 2000, Larsson *et al.* 1999b, Taherzadeh and Karimi 2007). The composition of the different phenolic compounds formed during pre-treatment varies and depend on both the plant source and the pre-treatment method (Larsson *et al.* 1999b). In general, the resulting mix is usually made up of phenolic acids, phenolic aldehydes, phenolic alcohols and phenolic ketones all of which are inhibitory to cells. A typical spruce hydrolysate will often consist of the phenolic compounds listed in Table 1.

The occurrence of phenolic compounds with various functional groups like aldehydes, acids, ketone and alcoholic, and the abundance of phenolic compounds in wood hydrolysates present major challenges to studying them in detail. In some studies aimed at understanding phenolic compounds, compounds having similar functional groups have been grouped together while representative compounds of each group were studied (Larsson *et al.* 2000), presumably under the assumption that compounds having the same functional group are similar in their inhibitory activities. It has been shown that the presence of phenolic compounds in hydrolysates may determine the fermentability of hydrolysates and directly impacts on ethanol productivity of *S. cerevisiae* (Larsson *et al.* 1999a, Larsson *et al.* 2000). The effects of many selected phenolic compounds and other inhibitors on yeast fermentative conditions have been screened, and strains of *S. cerevisiae* engineered for phenolic tolerance have been constructed and evaluated (Delgenes *et al.* 1996, Gregg and Saddler 1996, Larsson *et al.* 2000). It is known that certain phenolic compounds such as ferulic acid and vanillin can be assimilated and converted by *S. cerevisiae* (Clausen *et al.* 1994, Huang *et al.* 1993, Vanbeneden *et al.* 2008) however there are concentrations at which *S. cerevisiae* cannot survive the inhibition of such compounds, the various concentrations have not been defined for phenolic compounds.

Table 1 Table of phenolic compounds and the concentration range commonly found in spruce hydrolysates

Phenolic compounds	Amount (mg/L)
Gallic acid	7.1–10.2
Catechine	61–71.9
Vanillic acid	3.93–71.2
Syringic acid	42.3–42.87
Ferulic acid	42.91–45.08
Picein [3-(β-d-glucosyloxy)-hydroxy-acetophenone]	0.2–1.4
Pungenin[3-(β-d-glucosyloxy)-4-hydroxy-acetophenone]	0.2
Taxifoloin	2–33
Coniferyl aldehyde	35–301
Vanillic acid	0.01–35
Vanillin	36
4-hydroxybenzoic acid	39–81
Catechol	2
Acetoguaiacone	146
Trans cinnamic acid	10
Syringaldehyde	107

(Almeida et al. 2007b, Deflorio *et al.* 2011, Delvas *et al.* 2011, Evensen *et al.* 2000, Hutzler *et al.* 1998, Miyafuji *et al.* 2003).
Also, Pungenol (3',4'-hydroxy-acetophenone), Piceol (4'-hydroxyacetophenone), Trans-resveratrol, $_{p}$-Coumaric acid, Coumarins, Stilbenes, Styryl pyrones, Dihydroconiferyl alcohol, Hydroquinone, Homovanillic acid have all been found in various concentrations in spruce hydrolysates (Almeida et al. 2007b, Deflorio *et al.* 2011, Delvas *et al.* 2011, Evensen *et al.* 2000, Hutzler *et al.* 1998, Miyafuji *et al.* 2003).

Basing our experimental work on the hypotheses that (i) different phenolic compounds have different limits of toxicity on *S. cerevisiae* and (ii) mechanisms and activities of inhibition among phenolic compounds may be compound-specific, we have defined the toxicity limits of 13 different phenolic compounds selected from all classes of phenolic compounds commonly found in but not limited to spruce hydrolysates. We also studied the effects of the various phenolic compounds on the growth of *S. cerevisiae* and categorised the phenolic compounds into clusters based on their effects on growth. The influence of each cluster of phenolic compounds on metabolite yields were investigated in order to draw parallels and similarities between the phenolic compounds within each cluster and to explore whether compounds within each cluster have similar influence on the physiology of *S. cerevisiae*, all in order to better understand phenolic inhibition in lignocellulosic fermentation.

Materials and methods

Yeast strain

The industrial yeast strain *S. cerevisiae* Ethanol Red was used for this study. The yeast strain was obtained from the local wine-making and brewery shop in Gothenburg, Sweden.

Reagents

4-hydroxy-3-methoxycinnamaldehyde, homovanilyl alcohol, vanillin, syringic acid, vanillic acid, gallic acid, dihydroferulic acid, *p*-coumaric acid, hydroquinone, ferulic acid,

homovanillic acid, 4-hydroxybenzoic acid and vanillylidenacetone and other reagents used for growth media preparation in the studies were procured from Sigma-Aldrich.

Preparation of culture media

The medium used for all the cultivations was Yeast Minimal Mineral Medium (YMMM) (Verduyn *et al*. 1992). YMMM containing single phenolic substrates was prepared for each phenolic compound using the concentrations reported under "Results".

High throughput toxicity screening of phenolic compounds on *S. cerevisiae*

To define the range of values within which the toxicity limits of the compounds lie, high throughput toxicity screenings were done using Bioscreen C MBR (Oy Growth Curves Ab Ltd, Finland). Several concentrations of each phenolic compound were tested. Five replicates of each concentration step were run in parallel in the following conditions: T = 30°C ± 0.1; time = 96 hours; shaking speed setting = "maximum" optical density (OD) reading period = 15 min; wavelength filter = wideband 450 − 580 nm; initial OD = 0.1.

To facilitate data comparison, the readings obtained from the bioscreen were calculated back to standard spectrophotometric measurements at 600 nm via the formula:

$$OD_{spectro} = \frac{OD_{Bioscreen}}{Path\ \ Length(cm) \times 1.32} \quad (1)$$

Where

$OD_{spectro}$ = equivalent OD on spectrophotometer at 600 nm
$OD_{Bioscreen}$ = measured OD on the bioscreen

$$PathLength = \frac{volume\ (ml)}{r^2\ X\ \pi} \quad (2)$$

Where: volume = culture volume in a well in the bioscreen plate; r = radius of the well.

Non-linearity at higher cell densities was corrected as described by Warringer *et al.*, (Warringer and Blomberg 2003) using the formula:

$$ODcor = ODobs + \left(OD^2obs * 0.449\right) + \left(OD^3obs * 0.191\right) \quad (3)$$

Where: ODcor = the corrected OD and ODobs = the observed OD values, from which the average blank has been subtracted.

Aerobic batch cultivations were carried out in 100 ml or 250 ml baffled Erlenmeyer flasks (SIMAX, Czech Republic), containing 20 ml and 50 ml medium, respectively.

OD measurement of culture

Growth measurement for shake flask cultivations was done by measuring the turbidity of the culture at A_{600nm} using a Thermo Scientific GENESYS 20 Visible Spectrophotometer.

Determination of dry cell weight

Determination of Dry Cell Weight (DCW) was done in duplicates. Cells were harvested by filtration using predried and weighed filter paper discs of 0.45 μm pore size (Sartorius Stedim Biotech, Goettingen, Germany) on a water tap vacuum filter unit (Sartorius Stedim Biotech, Goettingen, Germany). The filter paper discs were dried in a microwave at 120 W for 15 minutes, weighed again and the biomass concentrated was calculated from the difference. DCW data were used for the calculation of biomass yield.

Analysis of metabolites

Analysis of ethanol, glycerol and acetate from the cultivation was performed by high performance liquid chromatography (HPLC) using a Dionex Ultimate 3000 HPLC unit (Thermo scientific, Dionex Corporation, Sunnyvale, USA) equipped with an Aminex HPX-87H (Biorad, USA) column of length 300 mm and diameter 7.8 mm, which was packed with 9 μm particles. A column temperature of 45°C was used for analysis and 5 mM H2SO4 was used as the mobile phase with a flow rate of 0.6 ml/min throughout the analysis. A Shodex RI-101 RI detector and an Ultimate 3000 VWD 3100 variable wavelength ultraviolet detector coupled to the HPLC unit were used to quantify the metabolites.

Determination yields

Yields (Yi/s) of ethanol, glycerol, acetate and biomass from the total consumed substrate (glucose) were calculated during the exponential growth phase by plotting each of the products against the total consumed glucose. The yield for each product was then obtained as the slope of the plot. Average values of biological replicates were used as the final yield for each culture condition.

Establishing concentration ladder of compounds

A concentration series was set up in increasing order for each compound to be screened for effect on *S. cerevisiae*. Since toxicity varies widely and using a universal concentration series for all of the compounds was not feasible, we determined consistent ratios of increase between consecutive points in the concentration series for all compounds to allow comparison of toxicity among the various compounds.

Determination of toxicity limits

The toxicity limits of the different phenolic compounds were determined based on the aspect (maximum specific

growth rates or final OD or elongation of lag phase) at which the yeast cultivations were most affected. The maximum specific growth rate of the *S. cerevisiae* in the presence of increasing concentrations of phenolic compounds were determined with increasing concentration of the compounds until cell growth stopped.

Statistical validation of data

All experimental data were subjected to Student t-test to determine the significance level with respect to the control. The number of replicates varied from 3 to 7, depending on the experiment. Therefore, t-tests for two-samples assuming unequal variances were performed with a significance level of probability set at $p < 0.05$. All error bars were standard deviations of multiple measurements of each parameter, all derived from biological replicates.

Results

Effect of compounds on *S. cerevisiae* growth pattern

We hypothesized that the physiological effect of each phenolic compound on *S. cerevisiae* would be unique and have phenotypic traits demonstrated in the growth pattern of *S. cerevisiae*. Yeast was grown in the presence of 4-hydroxy-3-methoxycinnamaldehyde, homovanilyl alcohol, p-coumaric acid, hydroquinone, ferulic acid, vanillin, syringic acid, homovanillic acid, 4-hydroxybenzoic acid, vanillic acid, gallic acid, vanillylidenacetone and dihydroferulic acid and the impact of the compounds on growth profiles, maximum specific growth rates and culture turbidity was assessed. *S. cerevisiae* had three unique growth patterns that distinctively grouped all the phenolic compounds into three clusters which we named cluster 1, cluster 2 and cluster 3 (Figure 1).

The culture containing phenolic compounds in cluster 1, exhibited lag phase elongation which increased with increasing concentration of phenolic compounds in the medium until a concentration of compound is attained at which growth was no longer possible. The second and third growth pattern clusters showed no elongation of lag phase. The phenolic compounds also had specific effects on maximum specific growth rate within their clusters. In cultures containing cluster 1 compounds, maximum specific growth rate decreased with increasing concentration of phenolic compound until a concentration is attained at which growth finally stopped (Additional file 1: Figure S1). Similarly, in cultures containing the cluster 2 compounds, the maximum specific growth rate reduced with increasing concentration of phenolic compound until a concentration is attained at which growth was no longer possible

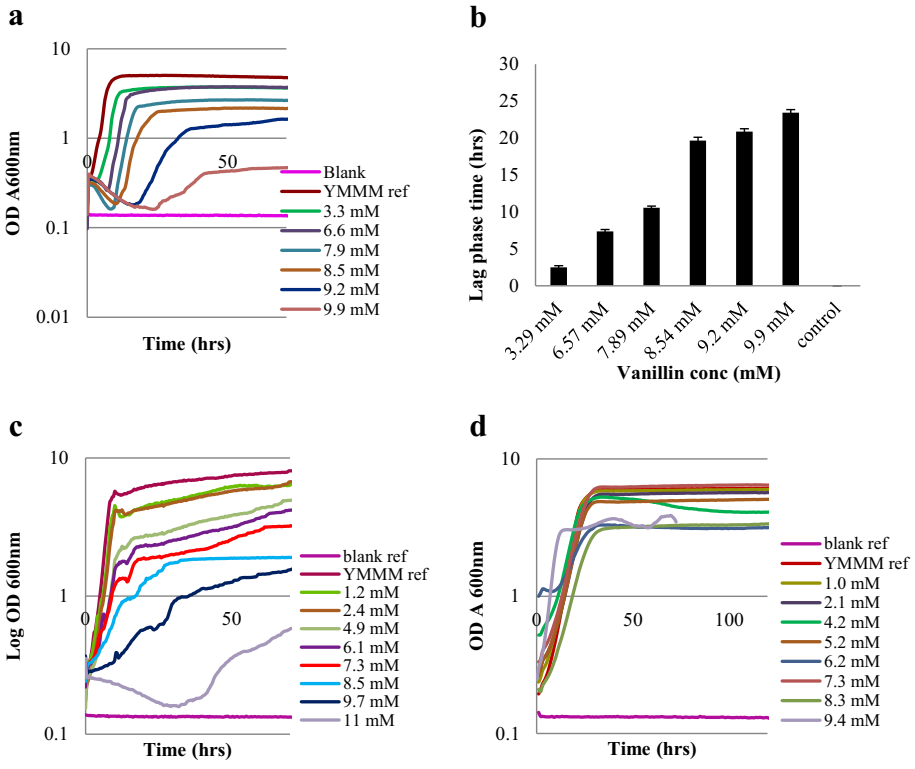

Figure 1 Three distinct growth profiles of *Saccharomyces cerevisiae* in the presence of phenolic compounds with: a. vanillin; c. *p*-Coumaric acid; d. Vanillylidenacetone representing common growth profiles within the three cluster groups. Elongation in lag phase was only shown in figure **b** for the first cluster since there was no lag phase elongation in the second and third clusters of compounds.

(Additional file 1: Figure S1b). In cultures contaning the third cluster of compounds however (as illustrated in Additional file 1: Figure S1c), the maximum specific growth rate remained constant until the concentration was attained at which growth was no longer possible.

The determination of biomass formation in the cultivations was limited to OD measurements on the bioscreen. In cultivations containing cluster 1 compounds, the OD of the yeast cultivations decreased with increasing concentration of phenolic compound until a concentration is attained at which growth stopped. As illustrated in Additional file 2: Figures S2a and S2, the reduction in OD was observed to be valid for the first and second clusters of compounds. In the third cluster however, although a reduction in the final OD was observed (Additional file 2: Figure S2c), the observed reduction was not as strong as in the first two compound clusters.

This categorization growth profile groups the thirteen phenolic compounds as;

- Cluster1: 4-hydroxy-3-methoxycinnamaldehyde, Homovanilyl alcohol, Vanillin, Syringic acid and Dihydroferulic acid.
- Cluster 2: p-Coumaric acid, hydroquinone, Ferulic acid, Homovanillic acid and 4-hydroxybenzoic acid and
- Cluster 3: Vanillic acid, Gallic acid and Vanillylidenacetone.

Different phenolic compounds have different limits of toxicity

In experimentally defining the concentration threshold at which the selected phenolic compounds completely inhibit yeast growth, we conducted toxicity screening on the phenolic compounds. During the screening, the maximum specific growth rates of the cultures when growth was last observed ranged between 0.07 h^{-1} and 0.09 h^{-1}, this was about 20% of the maximum specific growth rate of the control. In the presence of another set of phenolic compounds in which the cells experienced increased lag phase and reduced biomass with increasing concentration of the phenolic compounds, the concentration at which the cells last showed observable growth had an elongated the lag phase of about 5 times that of the control, the cells stopped growing in higher concentrations. In the third category, the cells suddenly stopped growing after a certain concentration and this concentration was noted. The toxicity screening revealed a wide range of toxicity among the compounds (Figure 2). The screening also revealed that each compound has a toxicity limit that is not necessarily based on its classification as an acid, alcohol, aldehyde or a ketone. Coniferyl aldehyde had the highest toxicity, becoming extremely toxic at 1.4 mM for cells to grow while syringic acid is the least toxic with cell growth continuing to be recorded at 22 mM. Further concentration increase in syringic acid was limited by strong interference in measurements as a result of the deep colouration of the medium (Figure 2).

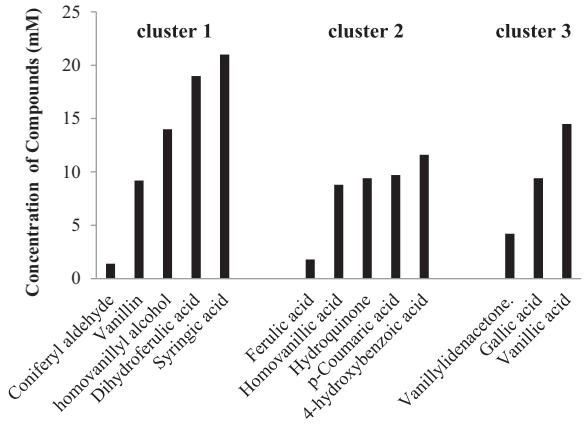

Figure 2 Bar chart showing concentrations at which different phenolic compounds within each growth-based cluster became too toxic for the growth of *Saccharomyces cerevisiae*.

Effects of toxic concentrations of phenolic compounds on ethanol and biomass titres and yields

In the next step we investigated whether compounds clustered together by order of growth pattern would also have similar effect on the yeast cell physiology. A pair of compounds was selected from each cluster and their effects on ethanol, acetate, glycerol and biomass yields were determined. Syringic and dihydroferulic acids were selected from the first cluster, homovanillic and 4-hydroxybenzoic acids were selected from the second cluster and vanillylidenacetone and gallic acid were selected from the third cluster. The compounds were added to the cultivation medium at their respective toxicity limit concentrations of 18.0 mM syringic acid, 18.0 mM dihydroferulic acid, 9.0 mM homovanillic acid, 11.0 mM 4 hydroxybenzoic acid, 4.2 mM vanillylidenacetone and 9.4 mM gallic acid.

Glucose consumption was particularly delayed in dihydroferulic acid cultivations (Figure 3). No significant difference in glucose consumption was observed between any of the cultures containing syringic, homovanillic, 4-hydroxybenzoic, gallic acid or vanillylidenacetone, and the control (Figure 3). Ethanol assimilation after glucose depletion during the respiratory growth phase was slowed down for all cultures with the phenolic compounds except for cultures containing syringic acid.

Further comparison within each cluster was done based on the yields of ethanol, glycerol, acetate and biomass. Ethanol yield in dihydroferulic acid and syringic acid cultures were similar at 0.43 ± 0.01 (g/g) and 0.38 ± 0.03 (g/g) respectively (Figure 4). The yield of glycerol in dihydroferulic

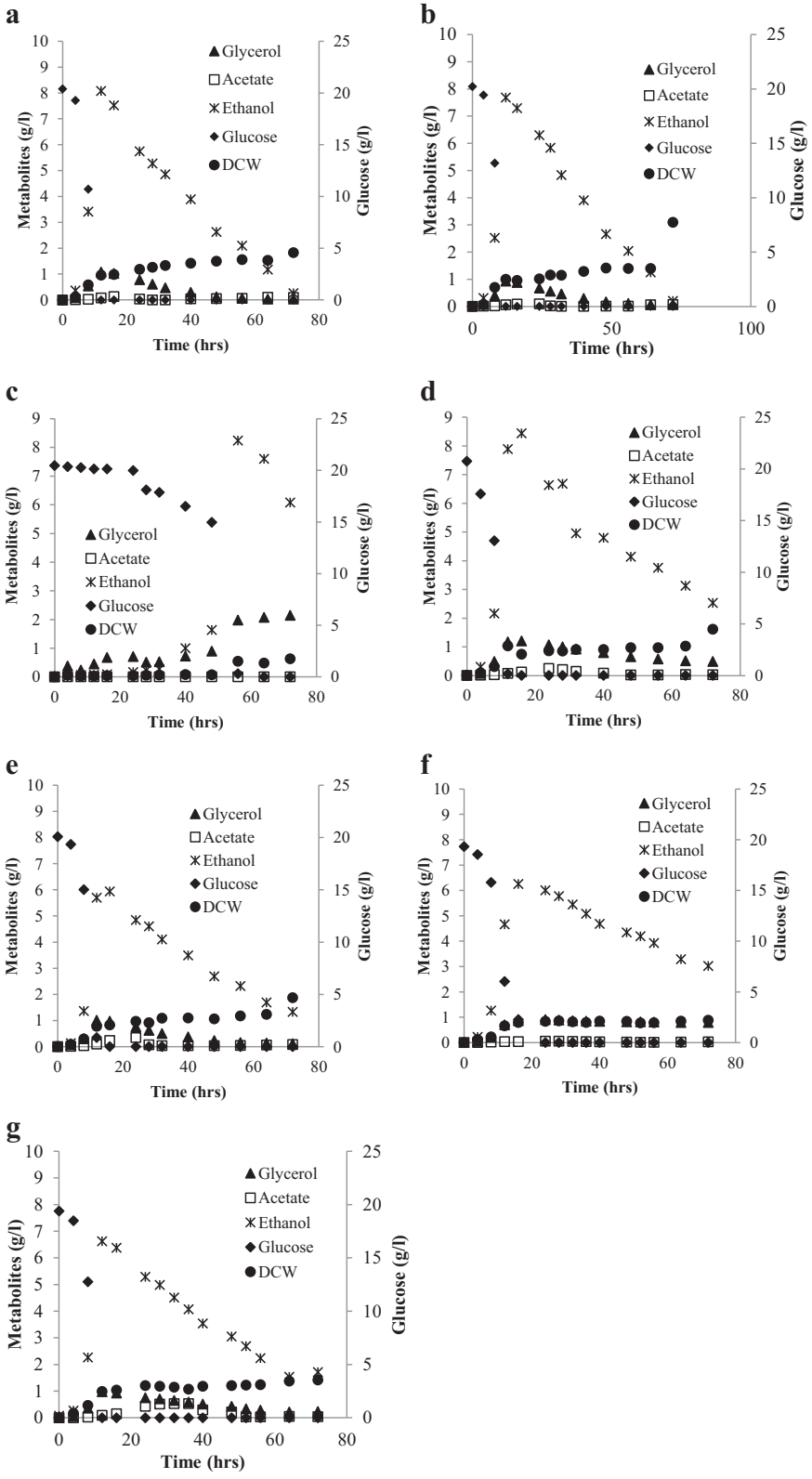

Figure 3 Metabolite profiles representatives of *Saccharomyces cerevisiae* cultivations in the presence of: a. YMMM; b. syringic acid; c. dihydroferulic acid; d. homovanillic acid; e. 4-hydroxybenzoic acid; f. vanillylidenacetone and g. gallic acid.

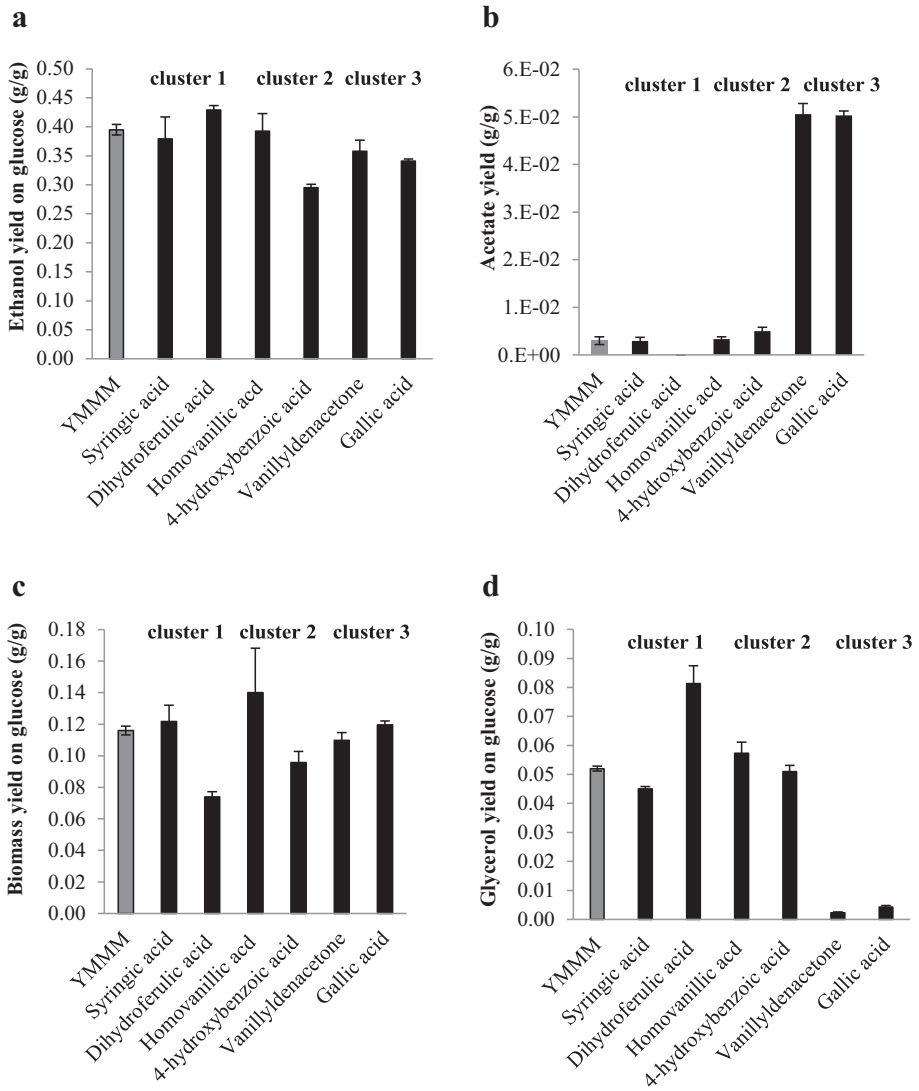

Figure 4 Intra-cluster comparison of the effects of phenolic compounds on: a. ethanol yield; b. acetate yield; c. biomass yield; and d. glycerol yield from glucose. Syringic and dihydroferulic acids were selected from cluster 1, homovanillic and 4-hydroxybenzoic acid from cluster 2, vanillylidenacetone and gallic acid from cluster 3.

acid containing cultures was higher than in syringic acid containing cultures with yields of 0.081 ± 0.006 (g/g) and 0.045 ± 0.001 (g/g) respectively. The most outstanding difference between this pair however was that acetate was not found in dihydroferulic acid cultivations while acetate yield was 0.003 (g/g) in syringic acid cultures which was the same as that of the control (Figure 4).

Ethanol, acetate and biomass yields in homovanillic acid cultures were significantly different to 4-hydroxybenzoic acid cultures. Ethanol yield at 0.39 ± 0.03 (g/g) and biomass at 0.14 ± 0.03 (g/g) were higher in homovanillic acid containing cultures compared with 0.3 ± 0.01 (g/g) and 0.096 ± 0.007 (g/g) respectively for ethanol and biomass yields in 4-hydroxybenzoic acid. Acetate yield was lower in homovanillic acid cultures at 0.003 ± 0.0005 (g/g) compared to 0.005 ± 0.001 (g/g) in 4-hydroxybenzoic acid. However, glycerol yields of homovanillic and 4-hydroxybenzoic acids were similar at 0.057 ± 0.004 (g/g) and 0.051 ± 0.002 (g/g) respectively. Results for vanillylidenacetone and gallic acid (cluster 3) proved very consistent for ethanol, biomass, acetate and glycerol yields (Figure 4). A significant difference was observed between glycerol and acetate yields in the third cluster (vanillylidenacetone and gallic acid) and those in the other two clusters and the control. Glycerol yield in cluster 3 was 10 times lower at 0.002 ± 0.0002 (g/g) for vanillylidenacetone and 0.004 ± 0.0006 (g/g) for gallic acid cultures, and acetate yield was higher by 10 times at 0.051 ± 0.002 (g/g) for vanillylidenacetone and 0.05 ± 0.001 (g/g) for gallic acid than in both YMMM and the other two clusters (Figure 4).

Overall ethanol yield in dihydroferulic acid was the highest at 0.43 (g/g) while 4-hydroxybenzoic acid had the lowest ethanol yield and the highest acetate yields of all cultures. The similarities in effect of each clustered pair of phenolic compounds on yeast metabolism indicate that compounds in the same cluster have similar inhibitory effects on yeast.

Discussion

In this study, we classified 13 different phenolic compounds commonly found in lignocellulosic hydrolysates according to their effect on *S. cerevisiae* growth. In particular, we showed that (i) the concentration that induces inhibitory effects is highly variable among phenolic compounds and it does not follow the order of phenolic aldehydes and ketones of being the most toxic, followed by acids and alcohols, respectively (Almeida *et al*. 2007a, Klinke *et al*. 2003) (ii) the influence of phenolic compounds on *S. cerevisiae* growth follows three major patterns; (iii) different compounds have distinct effect not only on biomass formation but also on the production of ethanol, acetate and glycerol.

Phenolic compounds have often been grouped and ordered as aldehydes, phenolic ketones, phenolic acids and phenolic alcohols, and their potency as inhibitors has largely been believed to reflect the same order. Phenolic aldehydes have generally been regarded as the most inhibitory while phenolic acids and alcohols tend to be seen as the least toxic (Almeida *et al*. 2007a, Klinke *et al*. 2003). In this study however, we demonstrated that the toxicity of phenolic compounds does not follow the assumed order in the subset of compounds we selected and is not dependent only on the recognised aldehyde, carboxylic acid, alcohol and ketone functional groups. Based on our results, we speculate that the inhibitory effects of phenolic compounds is a function of the combination of the occurrence of functional side groups (such as the methoxy and hydroxyl groups) and occurrence of unsaturated bonds in the structure of the compounds regardless of the categorization of the compounds as aldehydes, acid, alcohols or ketones. An example that supports this speculation is the different toxicities of coniferyl aldehyde (1.1 mM), ferulic acid (1.8 mM), and vanillin (9.7 mM) see Figure 5. Our results thus show that although coniferyl aldehyde is the most toxic at 1.1 mM, ferulic acid is more toxic at a toxicity limit of 1.8 mM than vanillin which is an aldehyde with a toxicity limit of 9.2 mM. The major difference between vanillin and coniferyl aldehyde is the occurrence of 2 extra carbon atoms sharing a double bond and linking the aldehyde group to the aromatic ring in coniferyl aldehyde. Ferulic acid also possesses 2 extra carbon atoms sharing a double bond and linking the carboxylic acid group to the aromatic ring. We speculate that these chemical features significantly

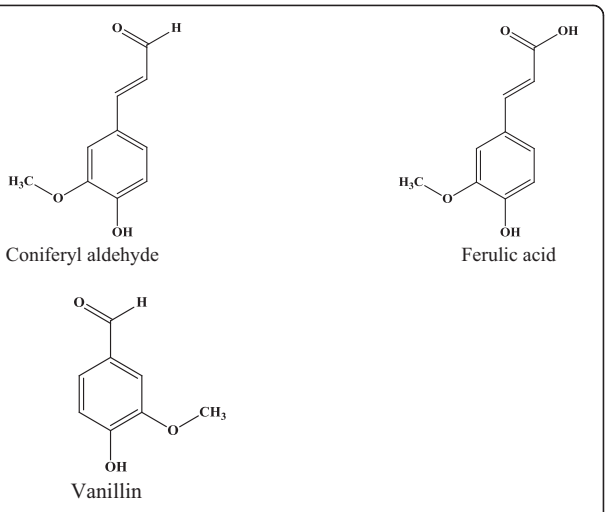

Figure 5 Structures of coniferyl aldehyde, ferulic acid and vanillin.

contribute to the toxicity of coniferyl aldehyde and ferulic acid, in line with earlier findings that the occurrence and positions of functional side groups as well as the presence of unsaturated carbon to carbon bonds influence the biological reactions and inhibitory activities of phenolic compounds in bacteria as well as their antioxidant activity in human (Ramaswam *et al*. 1972, Rice-Evans *et al*. 1996).

Three distinct growth patterns among the thirteen different phenolic compounds screened was observed, suggesting that compounds belonging to the same cluster display similarity in mechanisms of inhibition mechanisms. The similarity of ethanol yields between compounds representing cluster 1, acetate and glycerol yields in cluster 2 and the strong correlation of effects of vanillylidenacetone and Gallic acid (cluster 3) on ethanol, glycerol, acetate and biomass yields suggest that compounds belonging to the same cluster have similar inhibitory activity on yeast.

Phenolic compounds have been shown to reduce yields of ethanol and alter glycerol and acetate yields from *S. cerevisiae* fermentations. Studies by Ando *et al.,* (1986), revealed that syringaldehyde, m-hydroxybenzoic acid and vanillic acid did not inhibit ethanolic fermentation while coniferyl aldehyde led to poor fermentation and drastically reduced ethanol yield. Larsson *et al*. (2000) also corroborated the severely inhibitory effect of coniferyl aldehyde in their study. Our results aligned with previous studies which strengthens our confidence in the toxicity ranking of all our tested phenolic compounds in which we found coniferyl aldehyde to be the most toxic phenolic compound. The influence of the phenolic compounds on yeast physiology was mostly visible through their impacts on glycerol, biomass and acetate yields among the compound clusters. Absence of quantifiable acetate production, poor growth and delayed glucose utilisation characterized

the dihydroferulic acid cultivations. Known conditions that may prevent the accumulation of acetate in cultures include, disruption of acetaldehyde dehydrogenases, low formation of acetaldehyde coupled with effective oxidation of acetate by acetyl-coA-synthetase or the presence of low amount of glucose in cultures such that respirofermentative growth cannot take place (Postma *et al*. 1989). The presence of ethanol (0.43 g/g) and glycerol did confirm a respirofermentative growth for the yeast under these cultivation conditions. Speculatively, the apparent absence of acetate in the cultivation may resemble a situation where low activity of Cytosolic Mg^{2+} and Mitochondrial K^{+} acetaldehyde dehydrogenases Ald6p and Ald4p is present. Mutants of *ALD6* have been shown to substantially reduce acetate production while significantly increasing glycerol production. Double mutants of Ald6p and Ald4p have been shown to have delayed growth, and delayed glucose consumption (Remize *et al*. 2000), as observed in our dihydroferulic acid cultivation. It is therefore tempting to speculate that these two enzymes might be a direct or indirect target of ferulic acid, although this goes beyond the purpose of this article and deserves further investigation. Glycerol being a metabolite strongly associated with different types of stress in cells, the particularly high glycerol titre and yield in the cultivation of dihydroferulic acid is indicative of the cells being under significant stress from dihydroferulic acid although we have not defined the type of stress imposed at this stage of the study.

Although glucose consumption was delayed in dihydroferulic acid cultivations, ethanol yield was high and slightly superior to the ethanol yield in the control. Ethanol yields recorded in this study were high, ranging from 0.3 ± 0.01 (g/g) in 4-hydroxybenzoic acid cultures to 0.43 (g/g) in dihydroferulic acid cultures, we attribute this to the ability of the cells to adapt to the compounds and in certain cases convert some of them such as 4-hydroxybenzoic acid and dihydroferulic acid and eventually recover, thus bringing to attention and supporting findings of the natural ability of *Saccharomyces cerevisiae* to tolerate phenolic compounds (Stratford *et al*. 2007).

In conclusion, different phenolic compounds often present in lignocellulosic hydrolysates have toxicity limits that are not necessarily similar even between phenolic compounds sharing the same functional groups. An example of this would be the significant difference between ferulic acid and *p*-coumaric acid which we discovered in this study to respectively possess toxicity limits of 1.8 mM and 9.7 mM. The experiments showed that phenolics rich substrates may be fermentable since fermentability depends on the concentration and the nature of phenolic compounds present in them. Indications also emerged from the present study that mechanisms of inhibition among phenolic compounds are dissimilar and may not be defined by the classes of phenolic compounds (aldehydes, acids, alcohols and ketones) as they are currently known. Further studies involving investigation of gene regulation and varying enzymatic studies are needed to draw conclusions on the specificity of phenolic compounds inhibition in *Saccharomyces cerevisiae*.

Additional files

Additional file 1: Figure S1. Effects of increasing concentration of phenolic compounds on maximum specific growth rates with: a. vanillin; b. *p*-coumaric acid; c. vanillylidenacetone representing the effects of phenolic compounds in clusters 1, 2, and 3 respectively, created according to the observed growth profile.

Additional file 2: Figure S2. Effects of increasing concentration of phenolic compounds on final optical densities of cultivations with: a. vanillin; b. *p*-coumaric acid; c. vanillylidenacetone representing the effects f phenolic compounds in clusters 1, 2, and 3 respectively, created according to the observed growth profile.

Competing interests
The authors of this work declare that they have no competing interests.

Authors' contributions
Experimental design, work and writing of the manuscript were carried out by PTA. Experimental design and the subsequent manuscript were reviewed by MA. and LO. All authors read and approved the final manuscript.

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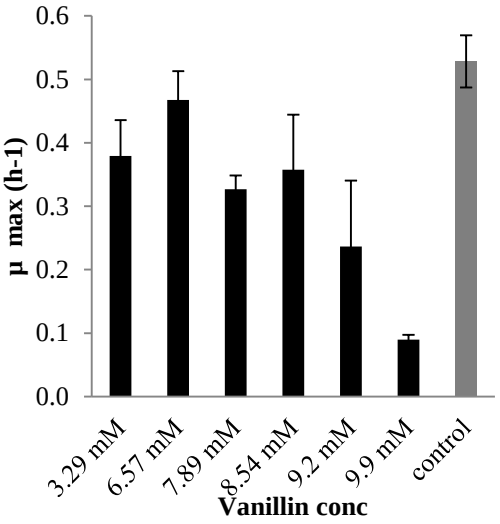

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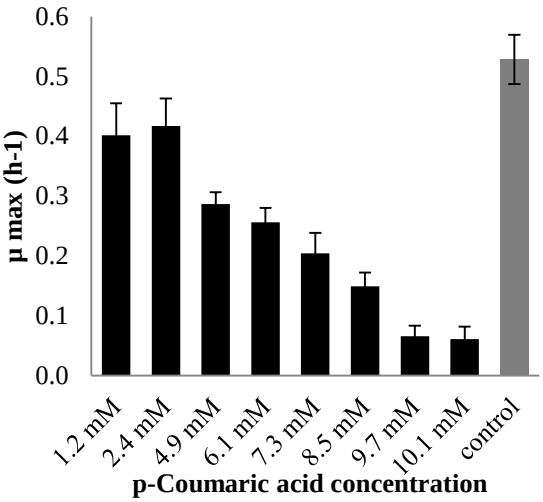

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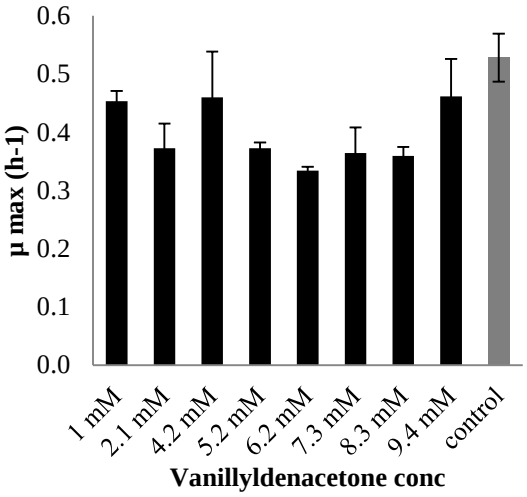

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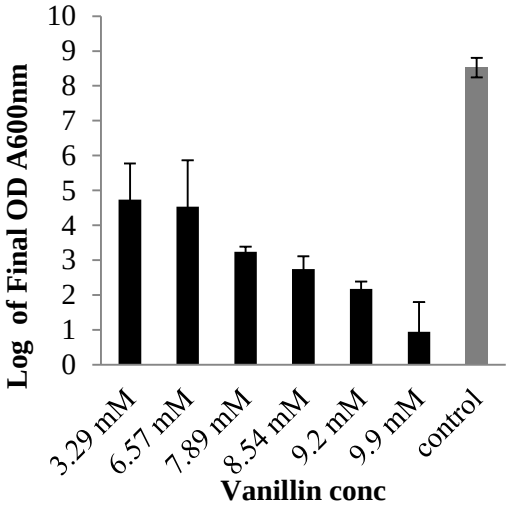
Figure S1. Effects of increasing concentration of phenolic compounds on maximum specific growth rates with: **a.** vanillin; **b.** *p*-coumaric acid; **c.** vanillylidenacetone representing the effects of phenolic compounds in clusters 1, 2, and 3 respectively, created according to the observed growth profile.



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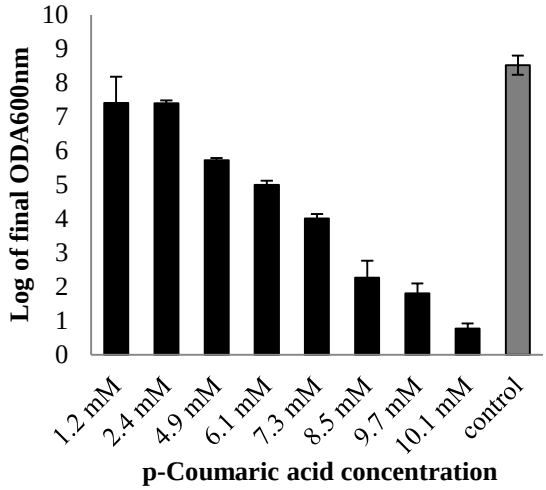

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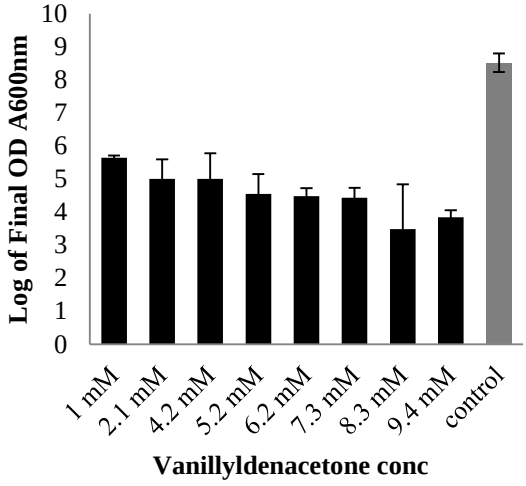

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Figure S2. Effects of increasing concentration of phenolic compounds on final optical densities of cultivations with: **a.** vanillin; **b.** *p*-coumaric acid; **c.** vanillylidenacetone representing the effects f phenolic compounds in clusters 1, 2, and 3 respectively, created according to the observed growth profile.

Paper II

Adeboye PT, Bettiga M, Aldaeus F, Larsson P, Olsson L. Catabolism of coniferyl aldehyde, ferulic acid and *p*-coumaric acid by *Saccharomyces cerevisiae* yields less toxic products. Microbial cell factories. 2015;14(1):149.

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MICROBIAL CELL FACTORIES

RESEARCH Open Access

Catabolism of coniferyl aldehyde, ferulic acid and *p*-coumaric acid by *Saccharomyces cerevisiae* yields less toxic products

Peter Temitope Adeboye[1], Maurizio Bettiga[1], Fredrik Aldaeus[2], Per Tomas Larsson[2] and Lisbeth Olsson[1*]

Abstract

Background: Lignocellulosic substrates and pulping process streams are of increasing relevance to biorefineries for second generation biofuels and biochemical production. They are known to be rich in sugars and inhibitors such as phenolic compounds, organic acids and furaldehydes. Phenolic compounds are a group of aromatic compounds known to be inhibitory to fermentative organisms. It is known that inhibition of *Sacchromyces cerevisiae* varies among phenolic compounds and the yeast is capable of in situ catabolic conversion and metabolism of some phenolic compounds. In an approach to engineer a *S. cerevisiae* strain with higher tolerance to phenolic inhibitors, we selectively investigated the metabolic conversion and physiological effects of coniferyl aldehyde, ferulic acid, and p-coumaric acid in *Saccharomyces cerevisiae*. Aerobic batch cultivations were separately performed with each of the three phenolic compounds. Conversion of each of the phenolic compounds was observed on time-based qualitative analysis of the culture broth to monitor various intermediate and final metabolites.

Result: Coniferyl aldehyde was rapidly converted within the first 24 h, while ferulic acid and *p*-coumaric acid were more slowly converted over a period of 72 h. The conversion of the three phenolic compounds was observed to involved several transient intermediates that were concurrently formed and converted to other phenolic products. Although there were several conversion products formed from coniferyl aldehyde, ferulic acid and *p*-coumaric acid, the conversion products profile from the three compounds were similar. On the physiology of *Saccharomyces cerevisiae*, the maximum specific growth rates of the yeast was not affected in the presence of coniferyl aldehyde or ferulic acid, but it was significantly reduced in the presence of *p*-coumaric acid. The biomass yields on glucose were reduced to 73 and 54 % of the control in the presence of coniferyl aldehyde and ferulic acid, respectively, biomass yield increased to 127 % of the control in the presence of *p*-coumaric acid. Coniferyl aldehyde, ferulic acid and *p*-coumaric acid and their conversion products were screened for inhibition, the conversion products were less inhibitory than coniferyl aldehyde, ferulic acid and *p*-coumaric acid, indicating that the conversion of the three compounds by *Saccharomyces cerevisiae* was also a detoxification process.

Conclusion: We conclude that the conversion of coniferyl aldehyde, ferulic acid and p-coumaric acid into less inhibitory compounds is a form of stress response and a detoxification process. We hypothesize that all phenolic compounds are converted by *Saccharomyces cerevisiae* using the same metabolic process. We suggest that the enhancement of the ability of *S. cerevisiae* to convert toxic phenolic compounds into less inhibitory compounds is a potent route to developing a *S. cerevisiae* with superior tolerance to phenolic compounds.

Keywords: Biorefinery, Phenolic compounds, Conversion, Coniferyl aldehyde, Ferulic acid, *p*-Coumaric acid

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Background

Lignocellulosic substrates are increasingly gaining attention as raw materials for biofuels and chemicals although numerous challenges on fermentability confront their usage as production platforms [1, 2]. Lignocellulosic substrates are primarily composed of cellulose, hemicellulose and lignin [3]. To disintegrate and make lignocellulosic biomass structurally accessible to enzymatic hydrolysis before fermentation, it is first subjected to a pre-treatment process [4, 5].

Also, the concept of chemical and fuel production in an integrated biorefinery is driving the interest in pulping process streams which are often rich in derivatives of lignin and hemicellulose [6, 7].

Pulping is a well-established technology for biomass disintegration and fractionation to make wood pulps [8]. Chemical pulping is a widespread process, the four classical methods principally used in chemical pulping are the kraft, sulfite, soda, and neutral sulfite semi-chemical pulping (NSSC) processes [9]. Pulping involves cooking wood biomass to obtain cellulose fibers during which delignification takes place and monomeric sugars from the hemicellulose fraction are released into the cooking liquor [10], the cooking liquor is then released as the process streams. Cooking liquor such as spent sulfite liquor, black liquor, delignification stream and pulp residues are useful energy and lignin sources, as well as having potentials for several purposes, including being used for bioethanol and chemical production [11]. In biofuel production, the acids and phenolic compounds derivatives of hemicellulose and lignin released into the process streams act as potent inhibitors against fermenting organisms [4, 12]. In the case of biochemical production, it has been shown that phenolic inhibitors in black liquor can be converted into value added chemicals [13].

The diverse nature of phenolic compounds present a significant challenge, they are thus the least studied and understood of all of inhibitors present in lignocellulosic materials [14]. Although studies have shown that various phenolic compounds such as ferulic acid and coniferyl aldehyde influence specific processes in *S. cerevisiae* [15, 16], the way the yeast cells respond and adapt to various phenolic compounds has not been well investigated. The ability of *S. cerevisiae* to convert particular phenolic compounds under fermentation, such as converting ferulic acid to 4-vinylguaiacol and coniferyl aldehyde to coniferyl alcohol, has been previously reported. Some *S. cerevisiae* strains with increased tolerance to the inhibitory activities of phenolic compounds were also engineered [17, 18]. However, several processes and mechanisms involved in the conversion of phenolic compounds in *S. cerevisiae* remain poorly understood. Information on the possible conversion pathway as well as a comprehensive list of products formed from the conversion is lacking. Apart from the importance of understanding the metabolic process involved with phenolic compound conversion, it is also important to investigate if the conversion products are more, equally, or less inhibitory in comparison with the parent compound. A conversion process that leads to less inhibitory compounds is one of the keys that could be explored for metabolic engineering strategies to develop a more phenolic tolerant *S. cerevisiae*. We have previously observed that inhibitory capacity of phenolic compounds against *S. cerevisiae* is compound specific, we also observed variation in the physiological influence on of phenolic compounds on *S. cerevisiae* [19].

In a lignocellulosic substrate, the different inhibitory compounds work in synergy and limit the chances to assign specific cell physiological response observed (effects) to the compounds inducing such a response. Although, the ability of *S. cerevisiae* to convert some phenolic, such as cinnamic acids have been previously reported [17, 20, 21], the complexity of lignocellulosic substrates and pulping streams makes it incredibly difficult to assign conversion products to specific starting compound during the bioconversion process. Therefore, monitoring the intermediates and products of catabolic conversion and investigating cell response to individual compounds may be best done by studying the effects of the phenolic compounds in a single substrate study. Based on this, we have done a selective study on the interaction of *S. cerevisiae* with three phenolic compounds coniferyl aldehyde, ferulic acid and p-coumaric acid under single substrate cultivation conditions in which only one of the three compounds is present in a cultivation set up.

In the present study, we closely investigated the interactions between yeast and phenolic compounds in a controlled environment, in order to understand the mechanisms and metabolic processes in *S. cerevisiae* which facilitate the conversion of, and resistance to, phenolic compounds. We have studied the conversion of phenolic compounds in order to provide information which is valuable for metabolic engineering and the development of yeast strains with improved tolerance to phenolic compounds. In addition, our investigation intends to pave the way to future research investigating the use of yeast as a catalyst for the potential aerobic conversion of phenolic compounds to chemicals of interest. In this paper, we present results detailing the individual metabolic conversion of three phenolic compounds by *S. cerevisiae*: coniferyl aldehyde, ferulic acid, and *p*-coumaric acid (Fig. 1). The results suggest that there is a previously unreported route that starts with phenolic aldehydes and leads to phenolic alcohols.

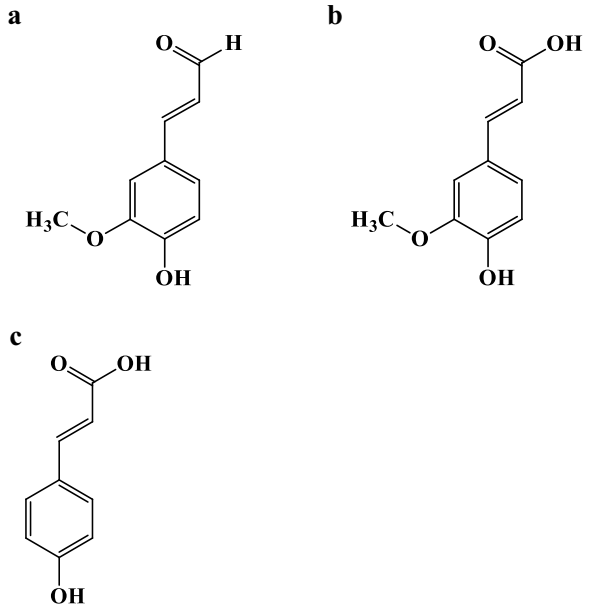

Fig. 1 Structures of; **a** coniferyl aldehyde, **b** ferulic acid and **c** *p*-coumaric acid

Results

Effects of coniferyl aldehyde, ferulic acid, and *p*-coumaric acid on cell growth

We have previously defined the toxicity limits of coniferyl aldehyde, ferulic acid, and p-coumaric acid on *S. cerevisiae* as 1.4, 1.8 and 9.7, respectively using high-throughput microtiter plate growth experiments [19]. The toxicity limits of the different phenolic compounds were defined as the concentration at which the cell performance is reduced by 80 % with respect to the control, and are based on the aspect of the yeast cultivations which were most affected (maximum specific growth rates, or final OD, or prolongation of the lag phase) [19]. In fermentor cultivations, it was found that the yeast cells did not grow in the presence of 1.4 mM coniferyl aldehyde. We therefore reduced the concentration of coniferyl aldehyde used in the cultivations by one concentration step to 1.1 mM in order to successfully cultivate the yeast cells in the presence of coniferyl aldehyde. To study the influence of coniferyl aldehyde, ferulic acid, and *p*-coumaric acid on *S. cerevisiae,* three cultivation experiments were set up. The first cultivation set up was with 1.1 mM coniferyl aldehyde in mineral medium, the second cultivation was with 1.8 mM ferulic acid while the third was with 9.7 mM *p*-coumaric acid. At these concentrations, the compounds did not arrest the growth of *S. cerevisiae*. The yeast grew at different specific rates in the presence of the different phenolic compounds, with the fastest growth being recorded in the presence of coniferyl aldehyde, closely followed by growth in the presence of ferulic acid. The slowest growth was observed in cultivations containing *p*-coumaric acid (Fig. 2). The maximum specific growth rates of the yeast under the influence of coniferyl aldehyde was $0.41 \pm 0.07\ h^{-1}$ while it was $0.35 \pm 0.02\ h^{-1}$ in ferulic acid. These were not significantly different from the specific growth rate of the control at $0.37 \pm 0.02\ h^{-1}$. However, the maximum specific growth rate of the cells in the presence of *p*-coumaric acid was statistically different, and was reduced to $0.29 \pm 0.02\ h^{-1}$. In the toxicity ranking carried out in the Bioscreen experiments [19], we observed that the maximum specific growth rates of *S. cerevisiae* were reduced by 80 % in comparison with the control cultivation when each of the phenolic compounds was present, we also observed a prolongation of the lag phase in the presence of coniferyl aldehyde. These were not observed in the bioreactor cultivation. We have attributed the changes in maximum specific growth rate and growth pattern to the scaling up of the experiment from the Bioscreen to bioreactors, which offer a different, better controlled cultivation condition. In the case of a pH related toxicity, which may well be among phenolic compounds, it is very probable that the differences in growth pattern between bioreactor cultivations and Bioscreen cultivations is pH related. The pH of all growth media was set to 5.0 at the start of each cultivation, however, the pH is not controlled in the bioscreen and reduces with time while in the bioreactor cultivations the pH was maintained at 5 throughout the cultivation.

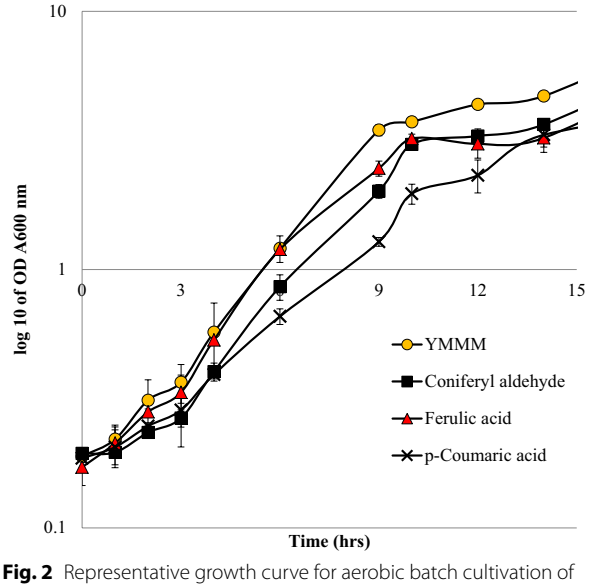

Fig. 2 Representative growth curve for aerobic batch cultivation of *S. cerevisiae Ethanol Red* in yeast minimal mineral medium (YMMM), coniferyl aldehyde, ferulic acid and *p*-coumaric acid

Effects of coniferyl aldehyde, ferulic acid, and *p*-coumaric acid on the titres and yields of fermentation products

During aerobic growth in batch cultures, *S. cerevisiae* induces aerobic fermentation during which, in addition to biomass, ethanol, glycerol and acetate are produced.

Biomass titres were 13.44 ± 0.06 g/l, 9.41 ± 0.05 g/l, 8.19 ± 0.02 g/l and 10.21 ± 0.03 g/l in cultivations containing coniferyl aldehyde, ferulic acid, *p*-coumaric acid and the YMMM control, respectively (Fig. 3). The biomass yields on glucose were 0.08 ± 0.009 g/g, 0.06 ± 0.008 g/g, 0.14 ± 0.07 g/g and 0.11 ± 0.019 g/g in cultures with coniferyl aldehyde, ferulic acid, *p*-coumaric acid and the YMMM control, respectively (Table 1).

As enumerated in Table 1, the ethanol yield was highest at 0.4 ± 0.01 g/g in cultures containing coniferyl aldehyde, while ethanol yields were 0.36 ± 0.005 g/g, 0.37 ± 0.011 g/g and 0.39 ± 0.011 g/g in cultures containing ferulic acid, *p*-coumaric acid and the YMMM control, respectively.

The glycerol yields were 0.08 ± 0.006 g/g in cultures with coniferyl aldehyde; 0.08 ± 0.002 g/g with ferulic acid; 0.12 ± 0.002 g/g with *p*-coumaric acid; and 0.08 ± 0.006 g/g in the YMMM control cultivation. The glycerol yield in *p*-coumaric acid was significantly higher than in other cultivations.

After the diauxic shift, at which point all the glucose has been consumed, ethanol, glycerol and acetate start to be assimilated. Assimilation of ethanol, glycerol and acetate was slowed in *p*-coumaric acid cultivations, the metabolites were still present after 73 h of cultivation, whereas they were assimilated within 50 h of cultivation in coniferyl aldehyde, ferulic acid, and in the control cultivations.

Conversion of phenolic compounds

Interestingly, we observed complete conversion of coniferyl aldehyde, ferulic acid and p-coumaric acid into other phenolic compounds. Conversion of the phenolic compounds was monitored through sampling and analysis of the culture broth at regular intervals during the course of the cultivations. Conversion of coniferyl aldehyde and ferulic acid was initiated by the cells within the first 2 h of cultivation, while the conversion of *p*-coumaric acid was first observed much later. After 24 h all the coniferyl aldehyde had been converted, while ferulic acid and *p*-coumaric acid required a period of over 72 h

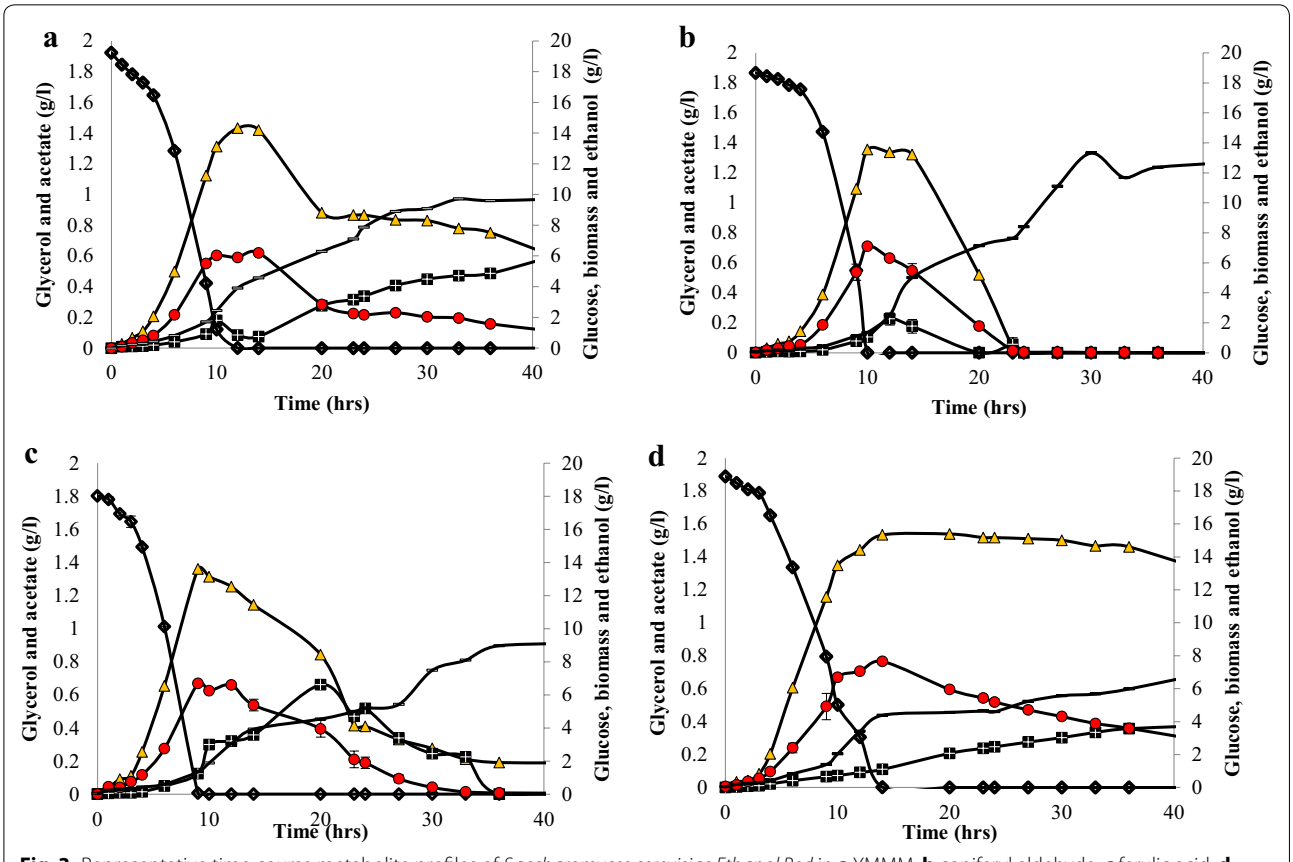

Fig. 3 Representative time course metabolite profiles of *Saccharomyces cerevisiae Ethanol Red* in **a** YMMM, **b** coniferyl aldehyde, **c** ferulic acid, **d** *p*-coumaric acid. (▲) glycerol, (■) acetate, (●) ethanol, (—) biomass, (◇) Glucose

Table 1 Metabolite profile of *S. cerevisiae* in control, phenolics-free yeast minimal mineral medium control medium in comparison with *S. cerevisiae* presence of each of 1.1 mM coniferyl aldehyde, 1.8 mM ferulic acid and 9.7 mM *p*-coumaric acid

	Titre at the end of cultivation (g/l)	Titre at the end of respirofermentative phase (g/l)	Yield (g/g)	μmax (h^{-1})	Respirofermentative q (gg^{-1} h^{-1})	Respiratory q (gg^{-1} h^{-1})
Yeast minimal mineral medium						
Glucose		0.01 ± 0.005	1	0.37 ± 0.02	3.94 ± 0.04	
Ethanol		6.87 ± 0.1	0.39 ± 0.011		1.53 ± 0.02	
Biomass	10.24 ± 0.03	2.13 ± 0.07	0.11 ± 0.019		0.37 ± 0.02	0.08 ± 0.001
Glycerol	0.06 ± 0.06	1.52 ± 0.1	0.08 ± 0.006		0.30 ± 0.05	
Acetate		0.37 ± 0.02	0.01 ± 0.001		0.03 ± 0.005	
CO_2	15.18 ± 0.03	2.99 ± 0.1	0.30 ± 0.012		1.56 ± 0.12	
Coniferyl aldehyde						
Glucose		0.04 ± 0.005	1	0.41 ± 0.07	4.68 ± 0.10	
Ethanol		5.73 ± 0.06	0.40 ± 0.01		1.87 ± 0.10	
Biomass	13.44 ± 0.06	1.39 ± 0.03	0.08 ± 0.009		0.35 ± 0.01	0.09 ± 0.005
Glycerol		1.18 ± 0.02	0.08 ± 0.006		0.37 ± 0.04	
Acetate		0.09 ± 0.04	0.01 ± 0.001		0.03 ± 0.002	
CO_2	16.25 ± 0.07	4.72 ± 0.03	0.34 ± 0.005		1.59 ± 0.12	
Ferulic acid						
Glucose		0.01 ± 0.005	1	0.35 ± 0.02	6.82 ± 0.08	
Ethanol	0.08 ± 0.02	6.57 ± 0.001	0.36 ± 0.005		2.44 ± 0.02	
Biomass	9.41 ± 0.05	1.35 ± 0.014	0.06 ± 0.008		0.41 ± 0.04	0.11 ± 0.006
Glycerol	0.18 ± 0.00	1.35 ± 0.05	0.08 ± 0.002		0.51 ± 0.04	
Acetate		0.12 ± 0.006	0.01 ± 0.001		0.05 ± 0.002	
CO_2	20.05 ± 0.1	3.34 ± 0.006	0.29 ± 0.01		2.29 ± 0.1	
p-Coumaric acid						
Glucose		0.02 ± 0.02	1	0.29 ± 0.02	2.95 ± 0.07	
Ethanol	0.02 ± 0.02	5.4 ± 0.05	0.37 ± 0.011		1.11 ± 0.05	
Biomass	8.19 ± 0.02	1.93 ± 0.05	0.14 ± 0.07		0.29 ± 0.02	0.09 ± 0.003
Glycerol	0.07 ± 0.03	1.32 ± 0.02	0.12 ± 0.002		0.31 ± 0.04	
Acetate		0.08 ± 0.01	0.01 ± 0.001		0.02 ± 0.008	
CO_2	12.23 ± 0.13	1.54 ± 0.0	0.03 ± 0.005		0.07 ± 0.004	

for complete conversion (Table 2). We observed the concurrent formation of several intermediates during the conversion. Some intermediates such as homovanillin, 2′,5′-dihydroxyacetophenone, coumaran and 3-vanilpropanol from coniferyl aldehyde were very transient, and were only present in the culture broth for a period of about 24 h, whereas other intermediate products, such as 4-vinylguaiacol from both coniferyl aldehyde and ferulic acid, as well as the ferulic acid intermediate from coniferyl aldehyde, were slowly converted into other products over a longer time period (Table 2).

During the first 2 h of cultivation, coniferyl aldehyde was initially converted to ferulic acid and ferulic acid isomer, before being further converted to other phenolic acids and other classes of compounds. Ferulic acid was also converted to ferulic acid isomer and dihydroferulic acid during the first 2 h of cultivation, before other conversion products were detected. The conversion trend in *p*-coumaric acid cultivations appeared to have fewer intermediates and products than in cultivations with coniferyl aldehyde and ferulic acid (Table 2). From the time evolution of the conversion products, it is evident that the observed conversion process was a sequential process involving several chemical reactions (Fig. 4). From the observed overlapping of products (Table 2), it is deducible that the chemical reactions involved in the conversion were simultaneously taking place.

Comparison of inhibition between coniferyl aldehyde, ferulic acid and *p*-coumaric acid their conversion products

To verify that the conversion of coniferyl aldehyde, ferulic acid and *p*-coumaric acid is a detoxification process, toxicity screening of several conversion products of each of the compounds was carried out and compared to that

Table 2 The conversion products profile of 1.1 mM coniferyl aldehyde, 1.8 mM ferulic acid and 9.7 mM *p*-coumaric acid with time

	0 h	2 h	24 h	48 h	72 h
Coniferyl aldehyde					
Coniferyl aldehyde	+	+	+		
Ferulic acid		+	+	+	
Ferulic acid, isomer		+	+	+	+
Dihydroferulic acid			+	+	+
Homovanillin			+		
2′,5′-Dihydroxyacetophenone			+		
Coumaran			+	+	
3-Vanilpropanol			+	+	
4-Hydroxyphenylethylethanol			+	+	+
Phenyl ethyl alcohol			+	+	+
4-Hydroxyphenylethanol			+	+	+
Benzoic acid, 3-methoxy-4-hydroxy			+	+	+
p-Coumaric acid			+	+	+
Benzenepropanoic acid			+	+	+
4-Vinylguaiacol			+	+	+
Benzeneacetic acid					+
Ferulic acid					
Ferulic acid	+	+	+	+	+
Ferulic acid, isomer		+	+		
Dihydroferulic acid		+	+		
2′,5′-Dihydroxyacetophenone			+		
5-Allyl-1-methoxy-2,3-dihydroxybenzene			+	+	
4-Hydroxyphenylethanol			+	+	+
Benzeneacetic acid			+	+	+
4-Vinylguaiacol			+	+	+
Phenylethyl alcohol			+	+	+
p-Coumaric acid					
p-Coumaric acid	+	+	+	+	+
Coumaran			+	+	+
4-Hydroxyphenylethylethanol			+	+	+
Phenyl ethyl alcohol			+	+	+
2,6-(1,1-Dimethylethyl)phenol			+	+	+

"+" connotes the presence of a compound while a blank space means the compound was absent

of their parent compounds. In the toxicity screening, the concentration at which each phenolic compound completely inhibits cell growth was determined similarly to what we had earlier reported [19]. We found that the conversion phenolic products were all less toxic than their parent compounds (Fig. 5). With conversion products such as phenyl ethyl alcohol, the toxicity limits were not reached. The experiment was terminated because of inaccuracy in the OD measurement caused by the strong interference from the colour of the compounds as well as the particulate background resulting from insolubility at higher concentrations. Phenyl ethyl alcohol did not inhibit yeast growth at 22.1 mM as effectively as 1.1 mM coniferyl aldehyde or 1.8 mM ferulic acid. Significantly higher concentrations of other conversion products such as vanillin, dihydroferulic acid, and coumaran, were also needed to inhibit yeast growth to a comparable extent to the coniferyl aldehyde, ferulic acid, and *p*-coumaric acid from which they were derived. This proves that the conversion products were much less toxic than their parent compounds and, therefore, the conversion serves as a detoxification process.

Discussion

Our results indicate that *S. cerevisiae* responds to phenolic-rich environment with processes which include

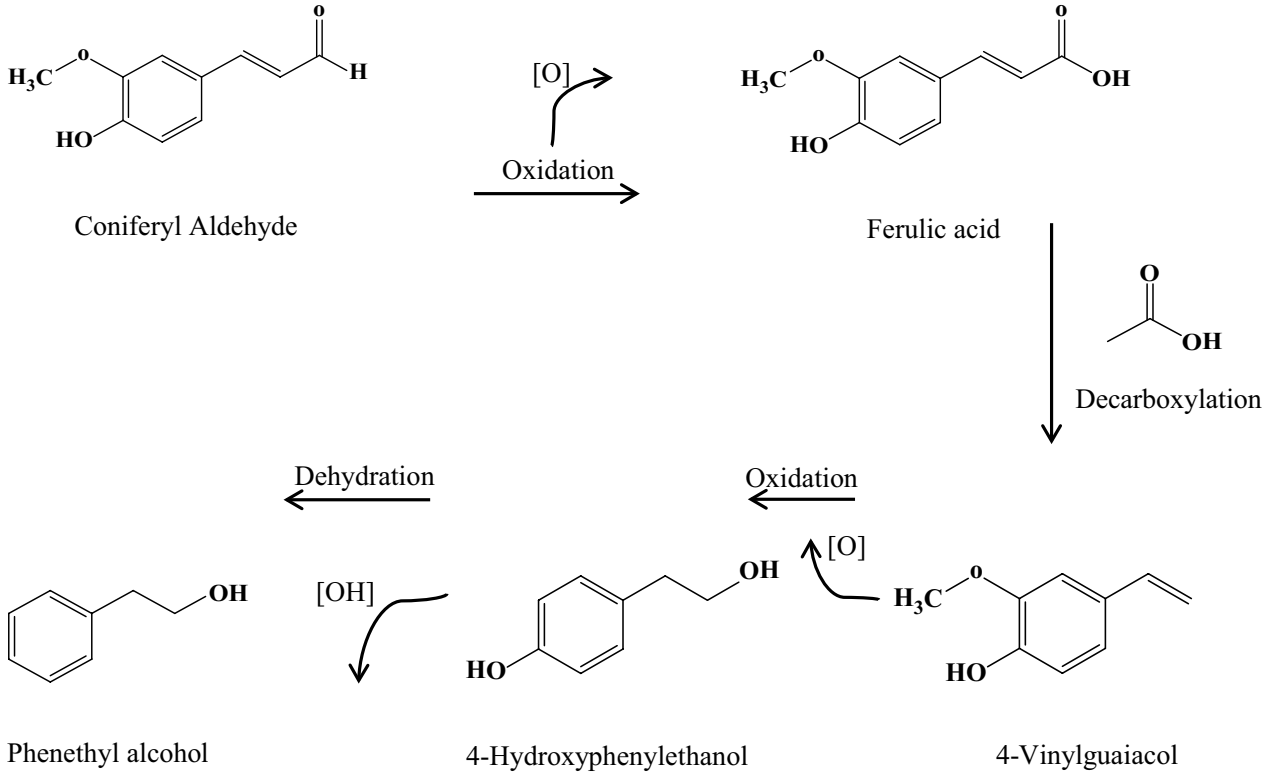

Fig. 4 A suggested conversion pattern in the detoxification of coniferyl aldehyde to phenyl ethyl alcohol, based on extracellular metabolites identified in the time evolution data presented in Table 2

conversion of the phenolic compounds, and that conversion could therefore be a possible mechanism for the cells to achieve tolerance to inhibitory compounds. In the present study, we showed that: (1) phenolic compounds are converted by *S. cerevisiae* and cell growth is not arrested during the conversion; (2) the conversion process of phenolic compounds is a sequential process with several intermediates, and may lead to detoxification since the conversion products are less toxic than their starting compounds; (3) some parts of the conversion pathway and mechanisms employed by *S. cerevisiae* may be common for all the phenolic compounds under investigation; (4) depending on the nature of the phenolic compounds involved, the conversion process may be rapid or slow.

In *S. cerevisiae*, the conversion and detoxification processes for handling many toxic substances leads to arrest of cell growth. Toxic metabolites, have also been known to arrest the growth of *S. cerevisiae*, mainly because they inhibit specific cellular processes inside the cell [22, 23]. Inhibitors such as furfural which are present in lignocellulosic materials have also been known to arrest growth and prolong the lag phase during conversion, severely affecting the cells redox metabolism, with potential impact on key cellular functions [24] In the present study, we observed a different relationship between growth and conversion of toxic compounds in *S. cerevisiae*. Simultaneous growth and conversion of the three phenolic compounds; coniferyl aldehyde, ferulic acid and *p*-coumaric acid was demonstrated in *S. cerevisiae*, even though the conversion was a detoxification process. Ahough previous studies have shown that coniferyl aldehyde causes a prolongation of the lag phase [19], the lack of lag phase prolongation may follow from the reduction of the concentration of coniferyl aldehyde from 1.4 to 1.1 mM during the scaling up of the process from the Bioscreen and Erlenmeyer flasks to the bioreactor, which, in combination with better aeration, agitation, and pH control in the bioreactor, may have favored yeast growth. The effect of the scale up to a bioreactor is also evident in the observation that the concentrations of compounds which resulted in a 80 % reduction in specific growth rate compared to the control in the Bioscreen-based screening, did not have the same level of inhibition in the bioreactor cultivation.

The most striking physiological differences between the inhibitor-containing cultivations and the -control were that the conversion of coniferyl aldehyde and that of ferulic acid similarly led to reduced biomass yields on glucose

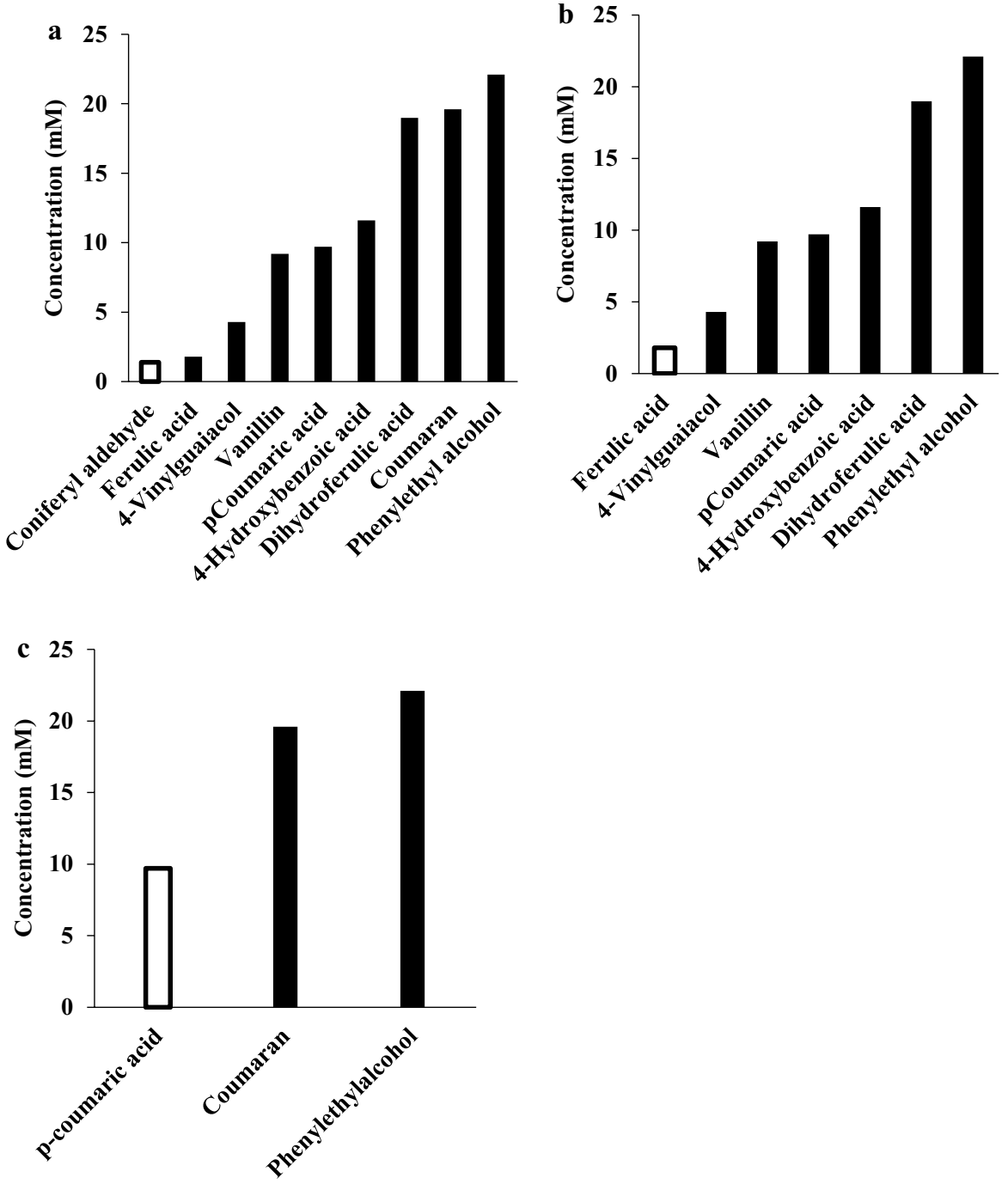

Fig. 5 Comparison of toxicity between conversion products (*filled bars*) and their corresponding parent phenolic compounds (*empty bars*): **a** coniferyl aldehyde and its products **b** ferulic acid and its products **c** *p*-coumaric acid and its products

in cultivations containing any of these two phenolic compounds; that increased glycerol accumulation was found in cultivations containing *p*-coumaric acid; and that ethanol yields are not reduced in the presence of any of these three phenolic compounds. Also, the conversion of coniferyl aldehyde as well as that of ferulic acid did not lead to a reduced maximum specific growth rate for the cells (Fig. 2). Coniferyl aldehyde may have favored an increased ethanol yield (Fig. 6a), however we do not yet fully understand the relationship—if any—between the

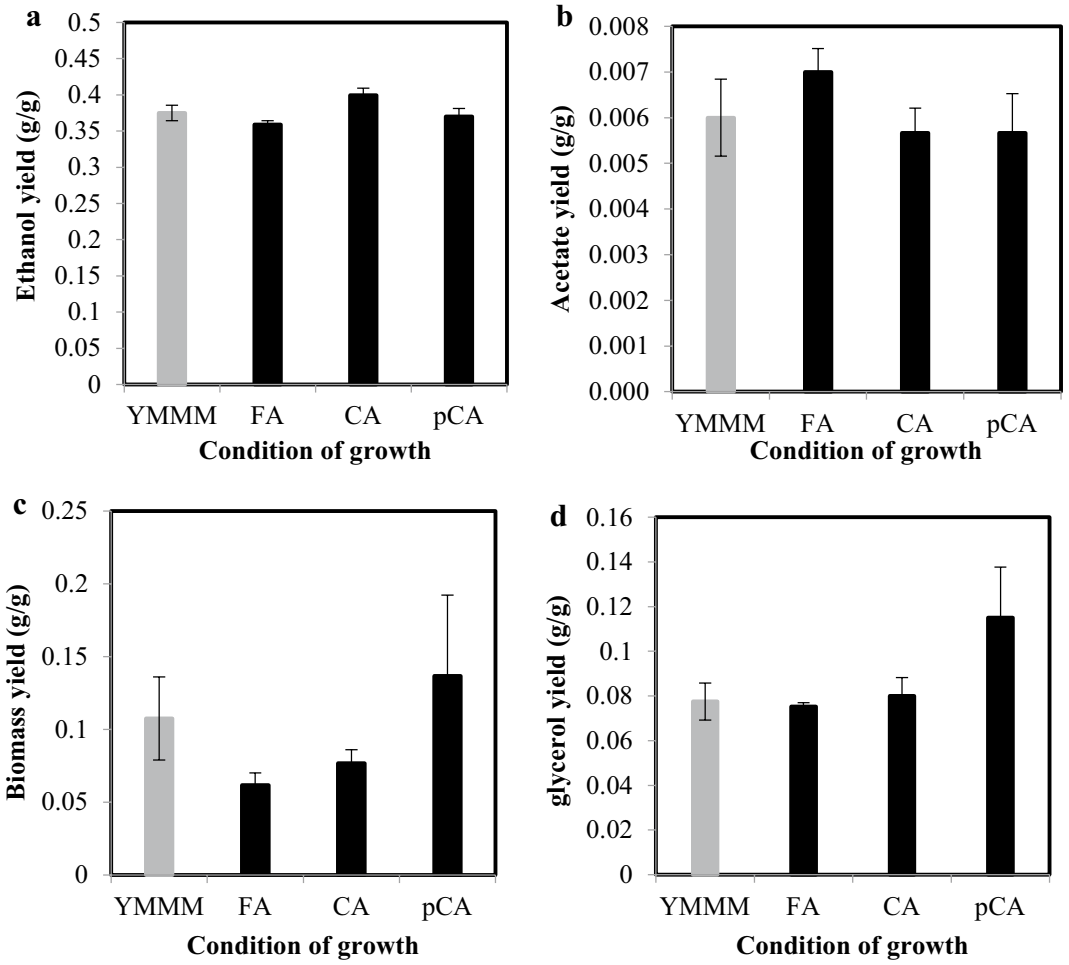

Fig. 6 Yields of: **a** ethanol, **b** acetate, **c** biomass and **d** glycerol on glucose at 14 h. n = 7 P < 0.05. *YMMM* yeast minimal mineral medium, *CA* coniferyl aldehyde, *FA* ferulic acid, *pCA p*-coumaric acid

increased ethanol yield with sub-lethal concentrations of coniferyl aldehyde observed in this study. Although we have not investigated molecular mechanism responsible for the increased ethanol yield and reduced biomass yield in the presence of coniferyl aldehyde, the phenomenon has also been observed in yeast under stressful cultivation conditions in some other instances, examples of which are a *Saccharomyces cerevisiae* strain with mutated *GPD1* which has been engineered for reduced glycerol production [25], another case was in a cultivation of *S. cerevisiae* under aliphatic acid stress [4].

The significant reduction in maximum specific growth rate observed in cultivations containing *p*-coumaric acid may suggest ATP usage when converting *p*-coumaric acid into its less toxic products. We speculate that certain ATP-dependent reactions are involved in the conversion of *p*-coumaric acid. The reduction in biomass formation and increased glycerol production in cultivations containing *p*-coumaric acid may be indicative of a difference between the mechanism employed by the cell to detoxify *p*-coumaric acid and that employed for coniferyl aldehyde and ferulic acid. Another interpretation could be that the compounds have different cellular targets and modes of inhibition in the cells. We speculate that this difference would aid interpretation of the results of our previous study, which showed that coniferyl aldehyde, ferulic acid, and p-coumaric acid, together with 10 other phenolic compounds, have different effects on *S. cerevisiae* growth, and, based on the different effects, belong to different clusters of phenolic compounds [19].

The results from this study enable us to hypothesize a conversion pathway that may be common for coniferyl aldehyde, ferulic acid, and *p*-coumaric acid, to further understand how *S. cerevisiae*, convert some phenolic

compounds such as ferulic acid earlier reported [18, 21]. The trend observed in the conversion process followed a transition from phenolic aldehyde to phenolic acid, after which phenolic alcohols and ketones were formed. Similarly, in the case of ferulic acid, an isomer of ferulic acid was formed, as well as dihydroferulic acid, before other compounds were formed. In the case of *p*-coumaric acid, there was a conversion directly to alcohols. This observed conversion trend, coupled with the commonality of conversion products among the three phenolic compounds studied, despite their structural differences, is indicative of a common conversion pathway for phenolic compounds in yeast. Different conversion intermediates were formed during the individual conversion of the three different phenolic compounds (Table 2) but they nevertheless lead to similar or the same conversion end products. Based on the conversion data, it is evident that the point at which the conversion begins is dependent on the toxicity and structural complexity of the starting phenolic compound. In general, we therefore hypothesize that the conversion pathway may hold true for other phenolic compounds in the sequence we have observed, with a phenolic aldehyde first being converted to one or more phenolic acids, and the phenolic acids then being converted to phenolic alcohols. Phenolic acids initially may be converted to other phenolic acids, but, invariably, all are converted to phenolic alcohols and other categories of phenolics, as illustrated in the simplified conversion scheme in Fig. 7. The conversion of coniferyl aldehyde to ferulic acid may require the activity of a coniferyl aldehyde dehydrogenase enzyme which is well known in bacteria species such as *Pseudomonas*, but has not been identified in *S. cerevisiae*. For the conversion we have observed under aerobic cultivation condition, we hypothesize that an oxidoreductase is responsible for the conversion of coniferyl aldehyde that we have studied, this would be further investigated in subsequent studies. It has been shown that the conversion of ferulic acid in *S. cerevisiae* is facilitated by decarboxylases [17, 20], the most popularly known being phenyl acrylic acid decarboxylase. In addition, we hypothesize also that alcohol acetyl transferases and alcohol dehydrogenases play

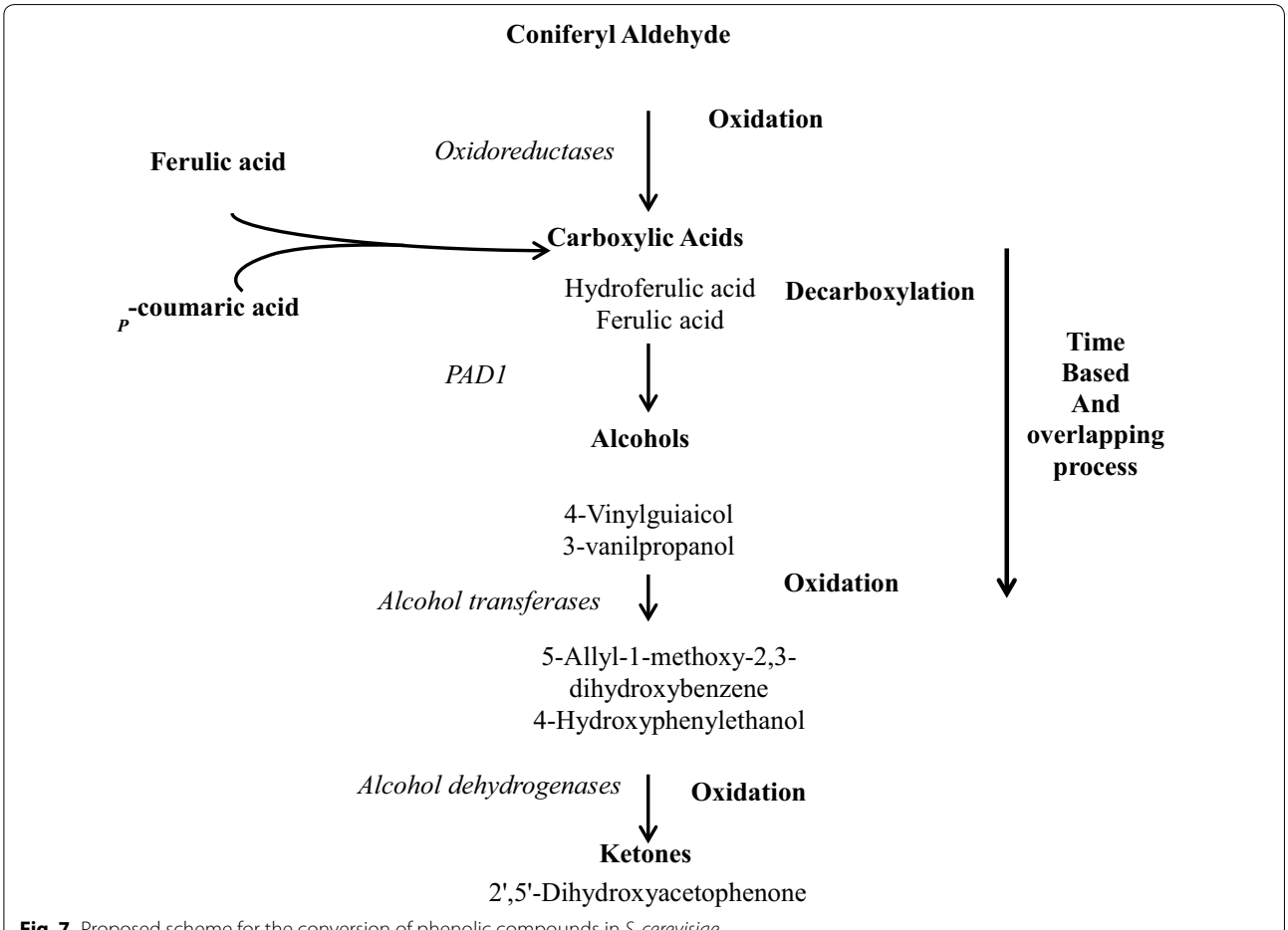

Fig. 7 Proposed scheme for the conversion of phenolic compounds in *S. cerevisiae*

active roles in the conversion of further conversion of phenolic alcohols to phenolic ketones. These hypothesis shall be investigated in our subsequent studies.

Another interesting observation is the isomerization of ferulic acid. While isomerization of phenolic compounds had previously been proposed in *S. cerevisiae* [21], to the best of our knowledge, this is the first time the formation of a ferulic acid isomer has been observed. The specific enzymes involved, and the benefit gained by forming isomeric intermediates are currently not clear. Among the three phenolic compounds tested, the conversion of coniferyl aldehyde—which is the most toxic compound—was observed to be the most rapid. Within the first 48 h, coniferyl aldehyde was completely converted into its intermediate products, while the conversion of ferulic and *p*-coumaric acids lasted for 72 h. To survive in a toxic phenolic environment, yeast cells undertake a detoxification process that converts toxic phenolic compounds to less toxic derivatives through the formation of several intermediates, until significantly less toxic compounds are formed.

The ability of the *S. cerevisiae* to convert, detoxify the phenolic compounds and produce high ethanol yields that is comparable to the control is an interesting observation because the *S. cerevisiae* strain used in this study is an industrial strain. It may be indicative of the relevance of the strain for second generation bioethanol production using substrates rich in phenolic compounds inhibitors.

Conclusion

We conclude that when *S. cerevisiae* is subjected to stress in a phenolics-rich substrate, *S. cerevisiae* responds by detoxifying its environment through the conversion of the toxic phenolic compounds, using a series of decarboxylation and oxidation processes into less toxic derivatives which the cells can then effectively cope with. This work highlights the in situ detoxification mechanisms in *S. cerevisiae* that can be exploited in developing phenolics resistant *S. cerevisiae* strains. Also, the close monitoring of the conversion process of coniferyl aldehyde, ferulic acid and *p*-coumaric acid as carried out in this study sheds light on the different stages of conversion and numerous intermediates formed in the process of detoxification of the phenolic compounds. Although the detailed metabolic pathway involved in this conversion process remains to be elucidated, the conversion explained in this study gives insight into the possibility of making high value phenolic compounds using *S. cerevisiae* as the cell factory. Although this is a single substrate study, through this work, we can however deduce that phenolic rich substrates such as pulping streams could be used for generating other products such as some of the phenolic conversion products which are useful for cosmetic, food and pharmaceutical applications. This therefore present an alternative use to lignocellulosic substrate other than production of biofuels.

Methods

Yeast strain

The industrial yeast strain *S. cerevisiae* Ethanol Red® (Fermentis, a division of S. I. Lesaffre, Lille, France) was used for this study.

Chemicals

All chemicals used in the preparation of the cultivation medium, including the phenolic compounds coniferyl aldehyde, ferulic acid, and *p*-coumaric acid, were purchased from Sigma-Aldrich GmbH, Germany.

All chemicals used in the chemical analyses of the starting phenolic compounds and their conversion products were of PA grade. Ethyl acetate, dichloromethane and acetone were purchased from Merck, Germany. 2,6-diethylnaphtalene and *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) were purchased from Sigma-Aldrich, Germany. *O*-Vanillin was purchased from Fluka, Sweden.

Medium preparation

The basal medium for the main cultivation was yeast minimal mineral medium (YMMM) [26]. Four cultivation media were used, (1) a control experiment without phenolic compounds in YMMM, (2) YMMM + 1.1 mM coniferyl aldehyde, (3) YMMM + 1.8 mM ferulic acid, and (4) YMMM + 9.7 mM *p*-coumaric acid. The concentration of phenolic compounds to be used in each medium had previously been determined by a toxicity experiment which has been reported previously [19].

Cultivation

Each cultivation condition was performed in triplicate. The inoculum was cultivated in Erlenmeyer flasks incubated at 30 °C and 200 rpm for a period of 18 h in YMMM. A volume of inoculum that resulted in an OD_{600} of 0.2 was added to the main cultivation. The main cultivations were carried out in DASGIP parallel bioreactor systems comprising of two units, each holding four SR0700ODLS vessels (DASGIP, Jülich, Germany). The culture volume was 700 ml and the fermentors were preconditioned overnight at pH 5. Aeration was set to 1 vvm at an impeller speed at 400 rpm. The cultivations were run for 96 h and air aeration was maintained at a flow of 11.7 l/h throughout the cultivation. A feedback loop was created between the impeller speed and the dissolved oxygen probe signal to maintain aeration above 40 % of oxygen saturation.

Cultivation of yeast was done separately in the presence of each phenolic compound.

Toxicity screening of phenolic compounds and conversion products on *Saccharomyces cerevisiae*

Experimental determination of the toxicity of the phenolic compounds and their conversion products was carried out by high-throughput toxicity screening using Bioscreen C MBR (Oy Growth Curves Ab Ltd, Finland), the set up was as we have described previously [19]. *S. cerevisiae* cultivations were done with different concentrations of single phenolic compounds in parallel. Growth was monitored in each cultivation and the concentration at which growth is not observed is noted. The toxicity limit for each phenolic compound is the concentration of a phenolic compound at which growth of the yeast is last observed. We have previously observed at this toxicity limit that the maximum specific growth rates and the final OD has been reduced to 80 % of the control, the elongation of lag phase is also 80 % more than that of the control.

OD measurement of culture

Growth was followed by OD_{600} measurements using a Thermo Scientific GENESYS 20 Visible Spectrophotometer for measurement of the optical densities of cultures.

Determination of dry cell weight

Determination of dry cell weight was performed in triplicate. 5 ml of culture was filtered using pre-dried and weighed filter paper discs of 0.45 μm pore size (Sartorius Stedim Biotech, Goettingen, Germany) on a water tap vacuum filter unit (Sartorius Stedim Biotech, Goettingen, Germany). The filter paper discs were dried in a microwave at 120 W for 15 min, weighed again and the biomass was determined from the difference.

Determination of specific growth rates

Maximum specific growth rates was calculated from the plot of the natural logarithm of the measured optical density of the cultivation against the time of the cultivations. For cultivations in Bioscreen, the readings obtained from the Bioscreen were calculated back to standard spectrophotometric measurements at 600 nm via the formula:

$$OD_{Spectro} = \frac{OD_{Bioscreen}}{PathLength\ (\text{cm}) \times 1.32} \quad (1)$$

where $OD_{spectro}$ = equivalent OD on spectrophotometer at 600 nm, $OD_{Bioscreen}$ = measured OD on the bioscreen

$$PathLength = \frac{volume\ (\text{ml})}{r^2 \times \pi} \quad (2)$$

where volume = culture volume in a well in the bioscreen plate; r = radius of the well.

Non-linearity at higher cell densities was corrected as described by Warringer et al. [27] using the formula:

$$OD_{cor} = OD_{obs} + (OD_{obs}^2 \times 0.449) + (OD_{obs}^3 \times 0.191) \quad (3)$$

where OD_{cor} = the corrected OD and OD_{obs} = the observed OD values, from which the average blank has been subtracted

Determination of rates and yields

The specific consumption rate of the substrate (glucose) was determined using the formula

$$q_{Substrate} = \frac{\mu}{Y_{(x/s)}} \quad (4)$$

where $q_{substrate}$ is the specific substrate consumption rate, μ the maximum specific growth rate, and $Y_{(x/s)}$ the biomass yield coefficient.

The specific productivity rates of biomass, ethanol, acetate and glycerol were calculated using the formula:

$$q_{product} = q_{Substrate} \times Y_{(p/s)} \quad (5)$$

where $q_{product}$ is the specific productivity rate, $q_{substrate}$ the specific substrate consumption rate, and $Y_{(p/s)}$ the product yield coefficient.

During the respiratory growth phase, the biomass yield $Y_{(x/s)}$, was calculated using a combination of glycerol, acetate and ethanol as substrate.

The yields of ethanol, glycerol, acetate and biomass from the consumed glucose were calculated during the exponential growth phase by plotting each of the products against the total consumed glucose. The yield for each product was obtained as the slope of a linear regression fitted to the plot. Average values of biological replicates were used as the final yield for each culture condition.

Analysis of metabolites

Analysis of metabolites from the cultivation was performed by high performance liquid chromatography (HPLC) using a Dionex Ultimate 3000 HPLC unit (Thermo Scientific, Dionex Corporation, Sunnyvale, USA) equipped with an Aminex HPX-87H (Biorad, USA) column (300 mm × 7.8 mm), packed with 9 μm particles. The column temperature was set to 45 °C, and 5 mM H_2SO_4 was used as the mobile phase at a flow rate of 0.6 ml/min. A Shodex RI-101 RI detector and a Ultimate 3000 VWD 3100 variable wavelength ultraviolet detector coupled to the HPLC unit were used to quantify the metabolites.

Time-based monitoring of the conversion of phenolic compounds and product formation

Simultaneously with the OD_{600} measurement, a 5 ml sample of culture was rapidly taken into 15 ml sample tubes and centrifuged at 0 °C and 5100 rpm for 5 min. Supernatants were kept frozen at −20 °C until qualitative analysis was carried out with gas chromatography–mass spectrometry (GC–MS).

Prior to GC–MS analysis, 0.5 ml of sample was mixed with 0.5 ml methyl acetate and 50 µl internal standard (100 µg/ml *o*-vanillin in ethyl acetate) and shaken. 0.45 ml of the mixture was dried using nitrogen until all the liquid had evaporated. 50 µl *N,O*-bis(trimethylsilyl) trifluoroacetamide (BSTFA) was then added, and allowed to react with the solid residue for 30 min at 80 °C. Finally, 950 µl dichloromethane and 50 µl external standard solution (111 µg/ml 2,6-diethylnaphtalene in acetone) was added.

The GC–MS analysis was performed using an Agilent HP7890A gas chromatograph (Agilent, Sweden) coupled with a Waters AutoSpec Premier magnetic sector mass spectrometer (Waters, UK). 1 µl of each sample was injected in splitless mode, and the injector temperature was held at 280 °C. Separation was carried out on a BPX5 capillary column (SGE Analytical Science, Sweden) of length 30 m, inner diameter 0.25 mm and film thickness 0.25 µm. Nitrogen with a flow of 1 ml/min was used as mobile phase. The temperature program was: 50 °C for 1 min, 10 °C/min to 300 °C, and then 300 °C for 10 min.

In the mass spectrometer, electron impact (EI+) was used for ionization. Mass spectra were recorded from m/z 40–400 with a total cycle time of 0.7 s. The resolution was 1000. Identification of the compounds with the highest abundance was performed by comparison of mass spectra with a NIST MS Search 2.0 library. The internal and external standards were used to determine tentative concentrations of the identified compounds.

Statistical validation of data

All experimental data obtained in the course of the experiment were subjected to the student t test to determine if there was a significance level of difference with respect to the control. The number of replicates varied from 3 to 7, depending on the experiment. Therefore, a t test for two-sample assuming unequal variances was performed, with a significance level of probability set at $p < 0.05$. All error bars are standard deviations from the averages of multiple measurements of each parameter, all derived from biological replicates.

Authors' contributions

PTA designed and performed the experiments and the writing of the article. MB and LO reviewed the experimental design, subsequent data and the manuscript. FA A qualitative analytical method for phenolic metabolites was set up and performed with the assistance of FA at Innventia AB. Discussion of analytical methods and data were carried out together with FA and TL. All authors read and approved the final manuscript.

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Compliance with ethical guidelines

Competing interests

The authors declare that they have no competing interests.

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Paper III

Adeboye PT, Olsson L, Bettiga M: A coniferyl aldehyde dehydrogenase gene from *Pseudomonas sp*. strain HR199 enhances the conversion of coniferyl aldehyde by *Saccharomyces cerevisiae*. Bioresource Technol 2016, 212:11-19.

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A coniferyl aldehyde dehydrogenase gene from *Pseudomonas* sp. strain HR199 enhances the conversion of coniferyl aldehyde by *Saccharomyces cerevisiae*

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HIGHLIGHTS

- Coniferyl aldehyde is inhibitory to *S. cerevisiae*.
- *CALDH* from *Pseudomonas* enhanced conversion of coniferyl aldehyde in *B_CALD*.
- *ALD5* deletion limits the capacity of *S. cerevisiae* to convert coniferyl aldehyde.

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ABSTRACT

The conversion of coniferyl aldehyde to cinnamic acids by *Saccharomyces cerevisiae* under aerobic growth conditions was previously observed. Bacteria such as *Pseudomonas* have been shown to harbor specialized enzymes for converting coniferyl aldehyde but no comparable enzymes have been identified in *S. cerevisiae*. *CALDH* from *Pseudomonas* was expressed in *S. cerevisiae*. An acetaldehyde dehydrogenase (Ald5) was also hypothesized to be actively involved in the conversion of coniferyl aldehyde under aerobic growth conditions in *S. cerevisiae*. In a second *S. cerevisiae* strain, the acetaldehyde dehydrogenase (*ALD5*) was deleted. A prototrophic control strain was also engineered. The engineered *S. cerevisiae* strains were cultivated in the presence of 1.1 mM coniferyl aldehyde under aerobic condition in bioreactors. The results confirmed that expression of *CALDH* increased endogenous conversion of coniferyl aldehyde in *S. cerevisiae* and *ALD5* is actively involved with the conversion of coniferyl aldehyde in *S. cerevisiae*.

1. Introduction

Aromatic compounds are the second most abundant class of organic compounds on earth, making up about 25% of the earth's biomass (Boll et al., 2002; Gibson and Harwood, 2002). Phenolic compounds play important roles in the interactions of plants with their abiotic environment (e.g. soil), and biotic environment, for example by attracting insects and serving as feeding deterrents to insects and birds (Harbourne, 1994). Phenolic compounds are also the building blocks of lignin which strengthens the structure of plants and also provides resistance to infection (Dorrestijn et al., 2000; Matern and Kneusel, 1988; Nicholson and Hammerschmidt, 1992).

Aromatic compounds are often inhibitory to microorganisms, thus limiting the possibility of bioconversion of these compounds. The ability of *Saccharomyces cerevisiae* to catabolize selected phenolic compounds has been reported and efforts have been made towards developing *S. cerevisiae* strains that exhibit increased tolerance to phenolic compounds by finding and expressing genes of interest in *S. cerevisiae* (Larsson et al., 2001; Sundström et al., 2010). Heterologous expression of genes from other organisms is a strategy that has been used to confer new traits on various microorganisms. Strains of *Escherichia coli* and *S. cerevisiae* have been successfully engineered to heterologously express genes that have conferred increased tolerance to phenolic compounds as well as the ability to metabolize them (Larsson et al., 2001; Overhage et al., 2003). The genes *vaoA* from *Penicillium simplicissimum*, *calA* and *calB*, encoding coniferyl alcohol dehydrogenase and coniferyl aldehyde dehydrogenase respectively, in the donor organism *Pseudomonas* sp. strain HR199, have been successfully expressed in the *E. coli* XLI-Blue strain to produce ferulic acid as an intermediate in the bioconversion of eugenol to vanillin.

To equip a strain for phenolic conversion, it is necessary to first identify and understand the genes involved in the conversion of

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phenolic compounds before overexpressing them in the host organism. Several bacteria and fungi have been found to be able to metabolize aromatic compounds, and are thus potential donors of genes that could be heterologously expressed in other microorganisms. The bacteria *Pseudomonas* sp. strain HR199 and *Corynebacterium sp*. are good examples of microorganisms known to be able to grow on eugenol as the carbon source (Achterholt et al., 1998; Rabenhorst, 1996; Tadasa, 1977) and this ability is being exploited to produce other phenolic metabolites from eugenol. It has been proposed that *Pseudomonas* sp. strain HR199 naturally converts eugenol via coniferyl alcohol, coniferyl aldehyde, ferulic acid, vanillin, and vanillic acid to produce protocatechuic acid (Achterholt et al., 1998; Priefert et al., 1997). *Corynebacterium* has been shown to employ a series of oxidation steps to metabolize eugenol, involving ferulic acid, vanillin and vanillic acid as intermediates before producing protocatechuic acid (Tadasa, 1977). Both *Pseudomonas* and *Corynebacterium* seem to use a set of oxidoreductases for the efficient conversion of eugenol, and some enzymes involved in the conversion in *Pseudomonas* have been identified such as the coniferyl aldehyde dehydrogenase (Achterholt et al., 1998; Tadasa, 1977) which primarily converts coniferyl aldehyde. Among several other phenolic compounds, coniferyl aldehyde is particularly potent in its inhibition of the growth of *S. cerevisiae*. Earlier, it was shown that coniferyl aldehyde is converted to several other phenolic metabolites (Adeboye et al., 2015). The aim of the present study was to enhance the ability of *S. cerevisiae* to convert coniferyl aldehyde by heterologous expression of an enzyme known to perform similar function in its native organism.

The utilization of plant biomass in technical and chemical processes often starts with the deconstruction and hydrolysis of the biomass (Wenzl, 1970). This leads to the breakdown of cellulose, hemicellulose and lignin in wood, yielding fermentable sugars, as well as several biologically active compounds that are inhibitory to the fermentative organisms used for second-generation biofuel and biochemical production. Together with organic acids from hemicellulose and furaldehyde from the dehydration of sugars, phenolic compounds from lignin significantly contribute to the microbial inhibition that limits the bioconversion of lignocellulose biomass (Larsson et al., 1999).

Since the coniferyl aldehyde dehydrogenase (CALDH) in *Pseudomonas* has been documented in literature to facilitate the catabolism of coniferyl aldehyde, a *S. cerevisiae* strain heterologously expressing CALDH from *Pseudomonas* sp. strain HR199 was engineered a goal to enhance its ability to catabolize coniferyl aldehyde. Coniferyl aldehyde was previously reported to be extremely inhibitory to *S. cerevisiae* (Adeboye et al., 2014), consequently an increased capacity to catabolize coniferyl aldehyde may also lead to increased tolerance of *S. cerevisiae* to coniferyl aldehyde.

Both *Corynebacterium* and *Pseudomonas* have been reported to possess efficient oxidoreductase enzymes that enable them to convert eugenol and intermediates like coniferyl aldehyde. Having observed the conversion of coniferyl aldehyde under aerobic batch cultivation of *S. cerevisiae*, it was proposed that *S. cerevisiae* would possess similar enzymes. A search for oxidoreductases that could be involved with the conversion of coniferyl aldehyde in *S. cerevisiae* was carried out with the aim of improving understanding of the catabolism of phenolic compounds by *S. cerevisiae*. Using the Basic Local Alignment Search Tool (BLAST) from the National Center for Biotechnology Information (NCBI) and the conserved domain data base at NCBI revealed acetaldehyde dehydrogenase *ALD5* to be the closest to Pseudomonas *CALDH*. The *ALD5* gene belongs to the aldehyde dehydrogenase family in *S. cerevisiae*.

A mutant strain of *S. cerevisiae*, *SC_ald5Δ*, in which the complete open reading frame of *ALD5* was deleted was subsequently engineered and investigated with regards to its sensitivity to and conversion of coniferyl aldehyde in order to test the hypothesis that *S. cerevisiae* possesses enzymes that are actively involved with the conversion of coniferyl aldehyde.

2. Materials and methods

2.1. Materials

2.1.1. Yeast strain

S. cerevisiae strains CEN.PK102-3A and CEN.PK113-7D were the parental strains used in this study. The strains developed in this study were *B_CALD*, *SC_ald5Δ* and the control. The genotypic characteristics of the strains used in this study are listed in Table 1.

2.1.2. E. coli

NEB 5-alpha Competent *E. coli* cells were used for plasmid construction. The competent cells were developed by New England Biolabs Inc. and were obtained from BioNordika, Sweden.

2.1.3. Chemicals

All chemicals used in the preparation of the cultivation medium, including coniferyl aldehyde were purchased from Sigma–Aldrich GmbH, Germany unless otherwise stated. All chemicals used in the GC–MS analyses were of PA grade. 2,6-diethylnaphtalene and N,O-bis(trimethylsilyl) trifluoroacetamide (BSTFA) were also purchased from Sigma–Aldrich, Germany . Ethyl acetate, dichloromethane and acetone were purchased from Merck, Germany and O-vanillin was purchased from Fluka, Sweden.

2.1.4. Protein sequence accession numbers

The nucleotide sequence systematic and accession numbers of the *ALD5* and *CALDH* genes are NP_010996, and WP_016502080, respectively.

2.2. Methods

2.2.1. Strain construction

TDH3 promoter amplified from digested plasmid DNA was used for *CALDH*. *TDH3* was amplified with the primer pair TDH3_fwd AAGCTTCAGTTCGAGTTTATCATT and TDH3_rev CTGCAGGTGTGTTT ATTCGAAAC. *CALDH* gene was codon optimized and synthesized by

Table 1
Strains of *Escherichia coli*, plasmid and *Saccharomyces cerevisiae* used and constructed in this study.

Escherichia coli background strain	Recombinant strain	Genotype	Source and reference
NEB 5-alpha Competent *E. coli*			New England Biolabs Inc.
Saccharomyces cerevisiae			
CEN.PK102-3A		*MATa ura3-52 leu2-3 MAL2-8c SUC2*	(Entian and Kotter, 2007)
CEN.PK113-7D		*MATa, MAL2-8 c, SUC2*	(Entian and Kotter, 2007)
CEN.PK113-7D	*B_CALD*	*MATa, MAL2-8 c, SUC2, CALDH*	This study
CEN.PK102-3A	*SC_ald5Δ*	*MATa, LEU, URA, ald5*	This study
CEN.PK102-3A	Control	*MATa, MAL2-8c SUC2, LEU, URA*	This study
Native plasmid		Character	Source and reference
YIplac128		*LEU*	(Gietz and Sugino, 1988)
YIplac211		*URA*	(Gietz and Sugino, 1988)

Genescript (California, USA). The *CALDH* gene was inserted in the *CAN1* locus of CEN.PK113-7D, the successful recombinant strain was the *B_CALD* strain. The insertion of *CALDH* was performed as described by Sambrook et al. (1989). The synthetic *CALDH* gene with *TDH3* was inserted by transformation, as a linear cassette in a double recombination event to replace *CAN1*. Selection of the *CALDH*-bearing strain was performed on canavanine plates containing a canavanine concentration of 80 μg/mL. The insertion was further confirmed by PCR using the following PCR protocol: denaturation at 95 °C for 3 min; 95 °C for 30 s, annealing at 48 °C for 39 s, elongation at 72 °C for 2 min over a cycle of 30 reactions. Final elongation was performed at 72 °C for 10 min and the holding temperature of the product was 10 °C.

ALD5 was deleted using CEN.PK102-3A as the background strain. The CEN.PK102-3A strain is auxotrophic for both *URA* and *LEU*, therefore *URA3* was chosen as the marker for the deletion of *ALD5*. Homologous recombination was used with the following primer pair; URA3ald5_fwd TAAGACAGAAAACTTCTTCACAACATTAA CAAAAAGCCAAAGAAGAAGAATTAGTTTTGCTGGCCGCATCTT and URA3ald5_rev ATGTCGAAAGCTACATATAAGGTTATCATACATACCTT CAATGAGCAGTCAACTCGGGCCTGAGTTACTTCA. The *URA3* template was amplified from the genomic DNA of *S. cerevisiae*. The subsequent PCR product was purified and transformed into CEN. PK102-3A. The deletion of *ALD5* was confirmed with a confirmation PCR using the following primer pair ald5_delcon_fwd GAATGGCTTCAAAGAACAGAAC and ald5_delcon_rev CACGAGGCAT TTTTCATTATTC. The strain was then subjected to a second round of transformation using the empty plasmid vector Yiplac 211 in order to make the strain fully prototrophic.

2.2.2. Medium preparation

The basal medium used for the main cultivation was the yeast minimal mineral medium (YMMM) (Verduyn et al., 1992). The bioreactor cultivation medium used contained 1.1 mM coniferyl aldehyde in YMMM. The concentration of coniferyl aldehyde used in each medium had been previously determined in a toxicity experiment (Adeboye et al., 2014). When screening for the concentrations tolerable to *S. cerevisiae*, the concentration of coniferyl aldehyde in the medium was varied from 0.67 mM to1.4 mM.

2.2.3. Cultivation of S. cerevisiae strains

The inoculum was cultivated in Erlenmeyer flasks incubated at 30 °C and 200 rpm for a period of 18 h in YMMM. Cells were harvested by centrifugation (3000 rpm for 5 min at room temperature) from a volume of inoculum with an optical density OD_{600} of 0.2. The cells were resuspended in fresh cultivation medium and immediately added to the main cultivation. The main cultivations were carried out in DASGIP® parallel bioreactor systems comprising of two units, each with four SR0700ODLS vessels (DASGIP, Jülich, Germany). The culture volume was 700 ml and the fermenters were preconditioned overnight at pH 5. Aeration was set to 1vvm at an impeller speed of 400 rpm. Cultivation was run for 96 h and aeration was maintained at 1vvm throughout the cultivation. A feedback loop was created between the impeller speed and the signal from the dissolved oxygen probe to maintain aeration above 40% of oxygen saturation. Triplicate cultivations were performed for each strain.

2.2.4. OD measurements for culture growth determination

Growth was monitored by measuring the absorption at 600 nm (OD_{600}) using a Thermo Scientific GENESYS 20 Visible Spectrophotometer.

2.2.5. Determination of dry cell weight

The dry cell weight was determined in triplicate using 5 ml of culture. The sample was filtered using pre-dried and pre-weighed filter paper disks with 0.45 μm pore size and a water-tap vacuum filter unit (both from Sartorius Stedim Biotech, Goettingen, Germany). The filter paper disks were dried in a microwave oven at 120 W for 15 min, weighed again, and the dry cell weight was determined from the difference in weight.

2.2.6. Determination of maximum specific growth rates

The maximum specific growth rate was calculated by plotting the natural logarithm of the measured optical density of the culture samples against time during cultivation. To determine the maximum specific growth rate on the Bioscreen, the readings obtained from the instrument were calculated back to standard spectrophotometric measurements at 600 nm using the expression:

$$\mathrm{OD}_{\mathrm{spectro}} = \frac{\mathrm{OD}_{\mathrm{Bioscreen}}}{\text{Path length} \times 1.32} \tag{1}$$

where: $OD_{spectro}$ = equivalent OD on spectrophotometer at 600 nm and $OD_{Bioscreen}$ = OD measured on the Bioscreen.

$$\text{Path length} = \frac{\text{volume (ml)}}{r^2 \times \pi} \tag{2}$$

where: volume is the culture volume in a well in the Bioscreen plate and r is the radius of the well.

Non-linearity at higher cell densities was corrected as described by Warringer et al. (Warringer and Blomberg, 2003) using the expression:

$$OD_{cor} = OD_{obs} + (OD_{cor}^2 * 0.449) + (OD_{cor}^3 * 0.191) \tag{3}$$

where: OD_{cor} is the corrected OD and OD_{obs} is the observed OD values, from which the average blank has been subtracted.

2.2.7. Determination of yields and rates

The yields of ethanol, glycerol, acetate, carbon dioxide and biomass from the consumed glucose were calculated during the exponential growth phase by plotting each of the products against the total consumed glucose. The yield for each product was obtained as the slope of a linear regression fitted to the plot. Average values of biological replicates were used as the final yield for each culture condition.

The specific consumption rate of the substrate (glucose) was determined using the relation

$$q_{\mathrm{substrate}} = \frac{\mu}{Y_{x/s}} \tag{4}$$

where $q_{substrate}$ is the specific substrate consumption rate, μ the maximum specific growth rate, and $Y_{(x/s)}$ the biomass yield coefficient.

The specific productivity of biomass, ethanol, acetate and glycerol were calculated using the relation:

$$q_{\mathrm{product}} = q_{\mathrm{substrate}} * Y_{p/s} \tag{5}$$

where $q_{product}$ is the specific productivity, $q_{substrate}$ the specific substrate consumption rate, and $Y_{(p/s)}$ the product yield coefficient.

During the respiratory growth phase, the biomass yield $Y_{(x/s)}$, was calculated using a combination of glycerol, acetate and ethanol as substrate. The average rate of conversion of coniferyl aldehyde was calculated by plotting the concentrations of coniferyl aldehyde against time, and determining the slope of the plot.

2.2.8. Toxicity screening of phenolic compounds and conversion products on S. cerevisiae

The toxicity of the phenolic compounds and their conversion products were determined experimentally by high-throughput toxicity screening using Bioscreen C MBR (Oy Growth Curves Ab Ltd, Finland), using the same set-up as described previously

(Adeboye et al., 2014). *S. cerevisiae* cultivations were performed in parallel with different concentrations of coniferyl aldehyde. Growth was monitored in each cultivation and the coniferyl aldehyde concentration at which growth was not observed was noted. The toxicity limit was defined as the highest concentration of coniferyl aldehyde at which growth of the yeast was observed.

2.2.9. Analytical methods

2.2.9.1. Analysis of metabolites. The fermentation metabolites produced during cultivation were analyzed using high-performance liquid chromatography (HPLC) using a Dionex Ultimate 3000 HPLC unit (Thermo Scientific, Dionex Corporation, Sunnyvale, CA, USA) equipped with an Aminex HPX-87H (Biorad, USA) column (column dimension, 1.3 × 7.8 mm, 9 μm particle size, 8% cross linkage). The column temperature was set to 45 °C, and 5 mM H_2SO_4 was used as the mobile phase at a flow rate of 0.6 ml/min. A Shodex refractive index detector (RI-101) and an Ultimate 3000 variable wavelength ultraviolet detector (VWD 3100) were used for quantification of the metabolites.

2.2.9.2. Time-based monitoring of the conversion of coniferyl aldehyde. Simultaneously sampling for growth measurement (OD measurement) at each time point, additional 5 ml volume of culture sample was rapidly collected, centrifuged at 0 °C and 5100 rpm for 5 min and the supernatants were stored at −20 °C until analysis was carried out with combined gas chromatography and mass spectrometry (GC–MS). Prior to GC–MS analysis, liquid–liquid extraction was carried out using 1 ml ethyl acetate to extract 1 ml of sample. Extraction was carried out at pH 2 in glass sample vials and 50 μl of an internal standard (100 μg/mL O-vanillin in ethyl acetate) was added to each sample. The samples were vortexed on a multi-tube vortex at 2000 rpm for 20 min. The samples were allowed to rest for 5 min after vortexing and derivatization was then performed. To derivatize the samples, 125 μl of the solvent phase (ethyl acetate) from the extracted sample was pipetted into GC–MS vial and 87.5 μl of a derivatization reagent mix consisting of 12.5 μl Pyridine, 0.75 μl Trimethylchlorosilane (TMCS) and 74.25 μl of N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) were rapidly added to each sample. The samples were capped and incubated in a rotary water bath at 70 °C and 120 rpm for 30 min. The GC–MS analysis was performed using DSQ II Single Quadrupole GC–MS chromatograph (Thermo scientific, Germany). One μl of each sample was injected in splitless mode, and the injector temperature was maintained at 250 °C. Separation was carried out using a DB-5 capillary column (Agilent, Sweden) of length 30 m, inner diameter 0.32 mm and film thickness 0.25 μm. Helium was used as mobile phase at a flow rate of 1 ml/min was used as mobile phase. The temperature program was: 50 °C for 1 min, 5 °C/min to 350 °C, and then 350 °C for 5 min.

Electron impact (EI+) was used for ionization in the mass spectrometer. Mass spectra were recorded from m/z 40 to 400 with a total cycle time of 0.7 s. The compounds with the highest abundance were identified by comparison of the mass spectra with an NIST MS Search 2.0 library. The internal and external standards were used to determine the concentrations of the compounds identified.

2.2.9.3. Preparation of cell free extracts for in vitro activity assays of Ald5 in engineered strains. The engineered *S. cerevisiae* strains were cultivated to mid-exponential phase with optical density of 3.0. Five ml of culture sample was taken, allowed to cool down on ice for 2 min and subsequently harvested by centrifugation at 4000 rpm for 5 min. The pelleted cells were washed twice in equivalent volume of 100 mM potassium phosphate ice cold buffer (pH 7.0). The cells were resuspended in 0.5 ml of 65 mM potassium phosphate buffer (pH 7.0) containing 1% concentration of protease inhibitor. The cells were subsequently disrupted using acid washed glass beads in a homogenizer using two disruption cycles of 6 m/s for 20 s with cooling of the samples on ice for 1 min between the two cycles. The cell debris and glass beads were removed by centrifugation at 1000 rpm for 5 min. The supernatants were transferred to clean tubes and immediately analyzed for aldehyde dehydrogenase activity.

2.2.9.4. Measurement of aldehyde dehydrogenase activity. K^+-activated aldehyde dehydrogenase was assayed using the method described by Postma et al. (Postma et al., 1989). The assay mixture contained 100 mM potassium phosphate buffer (pH 8.0), 15 mM pyrazole 0.4 mM dithiothreitol, 10 mM KCl and 0.4 mM NAD^+. A range of substrate concentrations and diluted enzymes and extract were first used to determine the points of substrate saturation (Vmax) after which 20 μl of cell free extract was used in a total reaction volume of 200 μl. The reaction was started with 1 mM acetaldehyde. 1 Unit of acetaldehyde dehydrogenase is defined as the amount that will oxidize 1.0 μmole of acetaldehyde to acetic acid per minute at pH 8.0 and at 25 °C in the presence of NAD^+.

2.2.9.5. In vitro analysis of coniferyl aldehyde conversion with cell free extracts. The assay mixture was the same as that of acetaldehyde dehydrogenase assay. The reaction mixture contained 100 mM potassium phosphate buffer (pH 8.0), 15 mM pyrazole 0.4 mM dithiothreitol, 10 mM KCl, 0.4 mM NAD^+ and cell extract. The reaction was started with 0.5 mM coniferyl aldehyde in a reaction volume of 1 ml in Eppendorf tubes incubated at 30 °C in a Thermomixer. Samples of 100 μl were drawn from the reaction at 15 min intervals. The samples were transferred into new Eppendorf tubes and the reaction was stopped by incubation in a Thermomixer at 90 °C for one minute. Each sample was subsequently cooled on ice, extracted with 100 μl of ethyl acetate, derivatized as described for other GC–MS samples and analyzed using GC–MS. Due to the nature of the substrate and analytical platform, the assay was not run at Vmax therefore, activity of the cell free on coniferyl aldehyde was intended as average on time and expressed as specific coniferyl aldehyde conversion rate (μmole/min/mg).

2.3. Statistical validation of data

All obtained experimental data were subjected to the student t-test to determine whether there were significant differences between the data obtained on the different strains. A two-sample t-test assuming unequal variances was therefore performed. The significance level was set at $p < 0.05$. All error bars indicate one standard deviation from the averages of multiple measurements of each parameter among biological replicates. Propagation of errors were also calculated among replicates by the expressions;

$$R = \frac{1}{3}\sum_{i=1}^{3} X_i = \frac{1}{3}(X_1 + X_2 + X_3) \tag{6}$$

$$\frac{\delta R}{R} = \sqrt{\left(\frac{\delta X_1}{X_1}\right)^2 + \left(\frac{\delta X_2}{X_2}\right)^2 + \left(\frac{\delta X_3}{X_3}\right)^2} \tag{7}$$

where R is the final result of averages, error in R is δR, X is each replicate experiment and δX is the deviation in each replicate.

3. Results and discussion

3.1. Acetaldehyde dehydrogenase activity in recombinant S. cerevisiae strains

To investigate the successful overexpression of *CALDH* in the B_*CALD* strain and the deletion of *ALD5* in the *SC_ald5Δ* strain, the acetaldehyde dehydrogenase activity was measured. The *B_CALD* extracts exhibited the highest activity per milligram of protein at 15.12 ± 0.17 mU/mg while the *SC_ald5Δ* had the lowest at 3.85 ± 0.08 mU/mg, which is consistent with the fact that it harbored *ALD5* deletion. The extract from the control strain (a prototrophic CEN.PK strain) displayed an intermediate specific activity of 4.78 ± 0.15 mU/mg. This confirmed the successful heterologous expression of *CALDH* in the *B_CALD* strain and a successful deletion of *ALD5* in the *SC_ald5Δ* strain.

3.2. In vitro conversion of coniferyl aldehyde

The conversion of coniferyl aldehyde was investigated in cell free extracts *in vitro* to determine the activity of the coniferyl aldehyde dehydrogenase gene in the *B_CALD* strain, and to study the role of Ald5 in the conversion of coniferyl aldehyde in *S. cerevisiae*. The trend followed by the three strains for *in vitro* coniferyl aldehyde conversion was the same trend as the one observed for acetaldehyde dehydrogenase activity measurements (Section 3.1). As expected, cell extracts from the *B_CALD* strain exhibited a specific coniferyl aldehyde conversion rate of 2.52 ± 0.05 μmole/min/mg while extracts from *SC_ald5Δ* and the control strain exhibited conversion rate of 0.71 ± 0.03 μmole/min/mg and 0.8 ± 0.01 μmole/min/mg, respectively. Thus, the highest coniferyl aldehyde conversion activity per milligram of protein (2.52 ± 0.05 U μmole/min/mg) was observed in the *B_CALD* strain, confirming the activity of CALDH on coniferyl aldehyde and its successful expression in the recombinant strain.

3.3. Tolerance to coniferyl aldehyde

Tolerance to coniferyl aldehyde was determined through a toxicity test in which all three strains were cultivated in the multiple automated growth curves instrument "Bioscreen" in the presence of different concentrations of coniferyl aldehyde. The tolerance was assessed by the ability of the cells to grow, and their maximum specific growth rates were calculated. As can be seen in Table 2, the maximum specific growth rates of the strains decreased with increasing concentration of coniferyl aldehyde, and at 1.4 mM, no growth was observed in any of the strains. At 1.18 mM coniferyl aldehyde, the *B-CALD* strain had a maximum specific growth rate of 0.083 ± 0.01 1/h while the *SC_ald5Δ* strain grew at 0.064 ± 0.01 1/h and the control strain at 0.098 ± 0.05 1/h. In terms of relative decrease in maximum specific growth rate, as compared with the mineral medium reference cultivations, *B_CALD* and the control both displayed a ~70% decrease, while *SC_ald5Δ* growth was more impacted by 1.18 mM coniferyl aldehyde, with almost 80% decrease in maximum specific growth rate. In light of these data, it is possible to conclude that the *B-CALD* strain retains the same tolerance to coniferyl aldehyde compared as the control, while the deletion of *ALD5* causes a more severe effect on growth.

3.4. Effect of conversion of coniferyl aldehyde on the physiology of the recombinant strains

The physiological performance of the strains varied when cultivated in the presence of 1.1 mM coniferyl aldehyde under aerobic batch cultivation conditions in instrumented bioreactors (Fig. 1). As expected, the strains displayed a slightly different growth behavior in these conditions compared to the high throughput test in Bioscreen (Section 3.3), most likely due to the different oxygenation conditions. Due to the redox nature of coniferyl aldehyde detoxification, and to the fact that the CALDH enzyme, overexpressed in the *B_CALD* strain, could deplete the cell of NAD^+ it can in fact be expected that oxygen limitation, and thus limited capacity of NAD(P)H re-oxidation, as experienced under cultivation in Bioscreen, has a negative impact on the *B_CALD* strain. All the strains experienced a lag phase in the presence of coniferyl aldehyde. The *B_CALD* strain experienced an exceptionally long lag phase of 36 h while strains *SC_ald5Δ* and the control had a lag phase of 14 h. The maximum specific growth rate of the *B_CALD* strain was 0.18 ± 0.02 1/h which is marginally lower than those observed in the *SC_ald5Δ* and the control strains which had growth rates of 0.24 ± 0.05 1/h and 0.24 ± 0.01 1/h respectively. The ethanol and glycerol yields did not differ between the strains, while the biomass, acetate and CO_2 yields varied between the strains and the control (Fig. 2). The *B_CALD* strain showed biomass yield of 0.14 ± 0.01 g/g, ~25% higher than the *SC_ald5Δ* and control strains for which the values were 0.11 ± 0.006 g/g and 0.11 ± 0.006 g/g respectively (Fig. 2b). The lower maximum specific growth rate of *B_CALD*, yet accompanied by a higher biomass yield, were reflected by a lower specific glucose consumption rate exhibited by the *B_CALD* strain was 1.32 ± 0.14 g/g/h while the *SC_ald5Δ* and the control strains showed specific glucose consumption rates of 2.18 ± 0.12 g/g/h and 2.2 ± 0.14 g/g/h respectively (Fig. 2f). Since the *B_CALD* strain exhibited a prolonged lag-phase and slower specific growth rate, the final biomass titer was lower and glucose consumption was slower in the strain. A lower specific glucose uptake rate favors respiration which in turn could have allowed a higher biomass yield on a glucose substrate rather than the aerobic production of ethanol. The lowest acetate yield, 0.005 ± 0.001 g/g was observed in the *B_CALD* strain, compared to *SC_ald5Δ* and the control strains where acetate yields were respectively 0.01 ± 0.001 g/g, 0.01 ± 0.001 g/g with the (Fig. 2c). The CO_2 yields in the strains *SC_ald5Δ* and the control strains were 0.70 ± 0.02 g/g, 0.75 ± 0.02 g/g respectively, while the *B_CALD* had the highest CO_2 yield at 0.83 ± 0.08 g/g (Fig. 2e). The high yield of CO_2 of the *B_CALD* strain is consistent with increased respiration, in line with the increased biomass yield on glucose.

It could as well be speculated that this may have been due to an increased energy demand in the cell, which resulted in increased respiration, to generate ATP required for the coniferyl aldehyde conversion process, rather than for growth. This strain converted coniferyl aldehyde before growth was initiated, unlike the other

Table 2
Maximum specific growth rates (1/h) of *B_CALD*, *SC_ald5Δ* and control strains at different concentration of coniferyl aldehyde.

Strain	Maximum specific growth rates (1/h) at different concentration of Coniferyl aldehyde					
	Blank medium	0.67 mM	0.84 mM	1.01 mM	1.18 mM	1.4 mM
B_CALD	0.27 ± 0.01	0.15 ± 0.03	0.13 ± 0.04	0.12 ± 0.02	0.08 ± 0.01	–
SC_aldΔ5	0.27 ± 0.02	0.22 ± 0.04	0.13 + 0.02	0.09 ± 0.01	0.06 ± 0.01	–
Control	0.29 ± 0.02	0.21 ± 0.03	0.21 ± 0.01	0.10 ± 0.03	0.10 ± 0.05	–

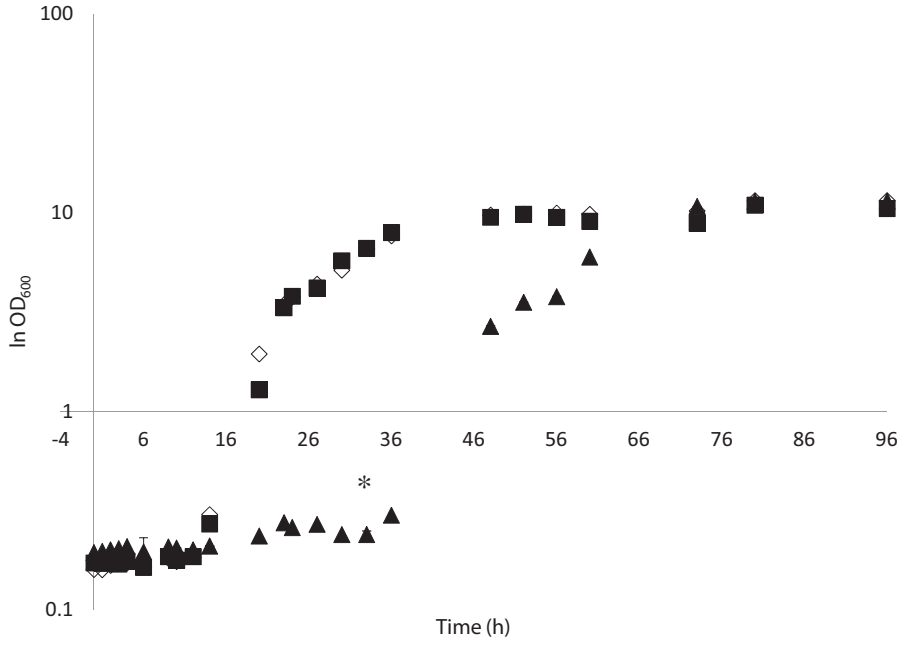

Fig. 1. Cell growth, expressed as the optical density in terms of the adsorption at 600 nm, of the investigated strains; *B_CALD* (▲), *SC_ald5Δ* (■), and the control strain (◆) in medium containing 1.1 mM coniferyl aldehyde. Asterisk denotes a significant difference between the *B_CALD* strain and other strains.

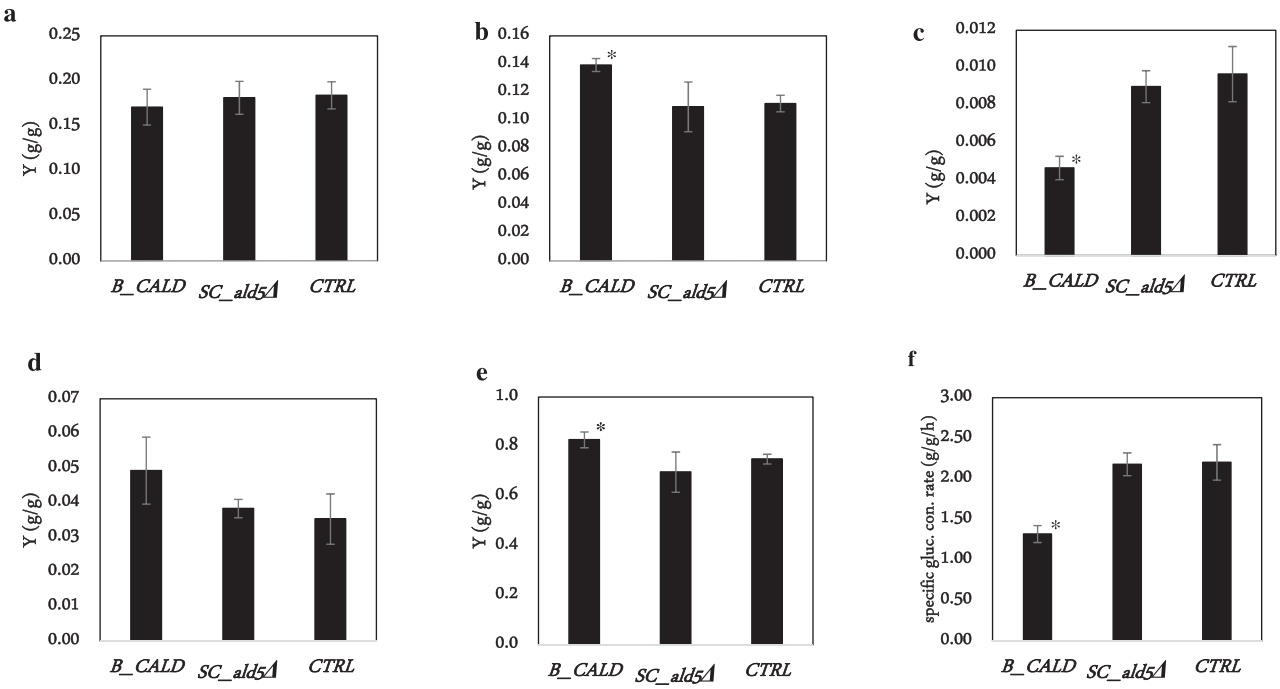

Fig. 2. Effect of 1.1 mM coniferyl aldehyde on the yields of (a) ethanol, (b) biomass, (c) acetate, (d) glycerol and (e) CO_2 from the cultivations of recombinant strains *B_CALD*, *SC_ald5Δ* and control using glucose as carbon source. (f) The specific glucose consumption rate. Asterisks denote differences between the *B_CALD* strain and other strains.

strains which grew and converted coniferyl aldehyde simultaneously. Furthermore, the reduction in acetate yield that was observed in the *B_CALD* strain may be directly related to the increased CO_2 yield. The observed change in the glucose consumption rate may be the reason for the decrease in aerobic fermentation, i.e. a limited Crabtree effect in the *B_CALD* strain were observed. This observation and the increased CO_2 yield both indicate increased respiration.

The prolonged lag-phase in the *B_CALD* strain was unexpected. It is known that during the conversion of some inhibitory compounds, cells may experience prolongation of lag-phase. A typical example is the conversion of HMF that leads to a prolongation of the lag-phase of *S. cerevisiae* (Ask et al., 2013). Prolongation of lag-phase during HMF conversion is due to the interference with the expression of several genes involved with biotransformation and detoxification of inhibitors, transcription factors and genes

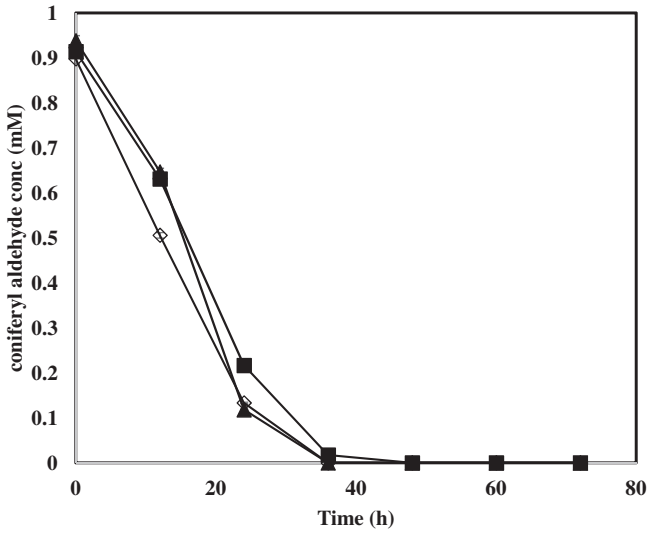

Fig. 3. Conversion of coniferyl aldehyde by the engineered strains *B_CALD* (), *SC_ald5Δ* (), and the control strain ().

that regulate pleiotropic drug response and genes involved with modification and degradation of damaged proteins (Ma and Liu, 2010). Factors similar to those responsible for the prolongation of lag-phase during the conversion of HMF have not been reported for *S. cerevisiae* in the presence of coniferyl aldehyde. Since a prolonged lag-phase was observed in the *B_CALD* strain expressing of *CALDH*, this is believed to be primarily due to the expression of *CALDH* which may have affected any of the cellular processes involved with growth. Considering the complexity of gene interactions and regulatory networks that are involved with growth, the molecular influence of the expression of *CALDH* in the *B_CALD* strain that led to a lag phase extension during conversion of coniferyl aldehyde is not understood. A hypothesis is that the *B_CALD* strain experienced a prolonged lag-phase because *CALDH* is expressed at high levels, and, at the initial high concentration of substrate (coniferyl aldehyde), it is could deplete the cell of NAD^+ for the conversion of coniferyl aldehyde, as hinted by the low acetate yield displayed by *B_CALD*. It has been shown that the heterologous expression of proteins often impact negatively on the specific growth rate and may not lead to an increase in cell performance because it overburdens and alters the host cell metabolism (Dürrschmid et al., 2008; Freigassner et al., 2009). The inability of the *B_CALD* strain to simultaneously grow and convert coniferyl aldehyde and the reduced specific growth rate may also be linked to expression mechanism of the *CALDH* enzyme in the new host, this was not investigated in this study.

3.5. Conversion of coniferyl aldehyde

Complete conversion of the 1.1 mM coniferyl aldehyde in the medium was observed in all the strains. The *B_CALD* and the control strains converted all the coniferyl aldehyde in 36 h while it took the *SC_ald5Δ* strain 48 h to completely convert all the coniferyl aldehyde (Fig. 3). Conversion started during the lag phase and all the strains experienced a prolongation in the lag phase compared to their cultivation in the absence of coniferyl aldehyde (Fig. 4). Cessation of growth for a period of 36 h was observed in the *B_CALD* strain during which period the conversion of almost all of the coniferyl aldehyde occurred. *SC_ald5Δ* and the control strains grew and converted coniferyl aldehyde simultaneously (Figs. 3 and 4).

The volumetric conversion rate of coniferyl aldehyde differed significantly between the three strains, as can be seen from Table 3. During the first 12 h average conversion rates of coniferyl aldehyde by strains *B_CALD*, *SC_ald5Δ* and the

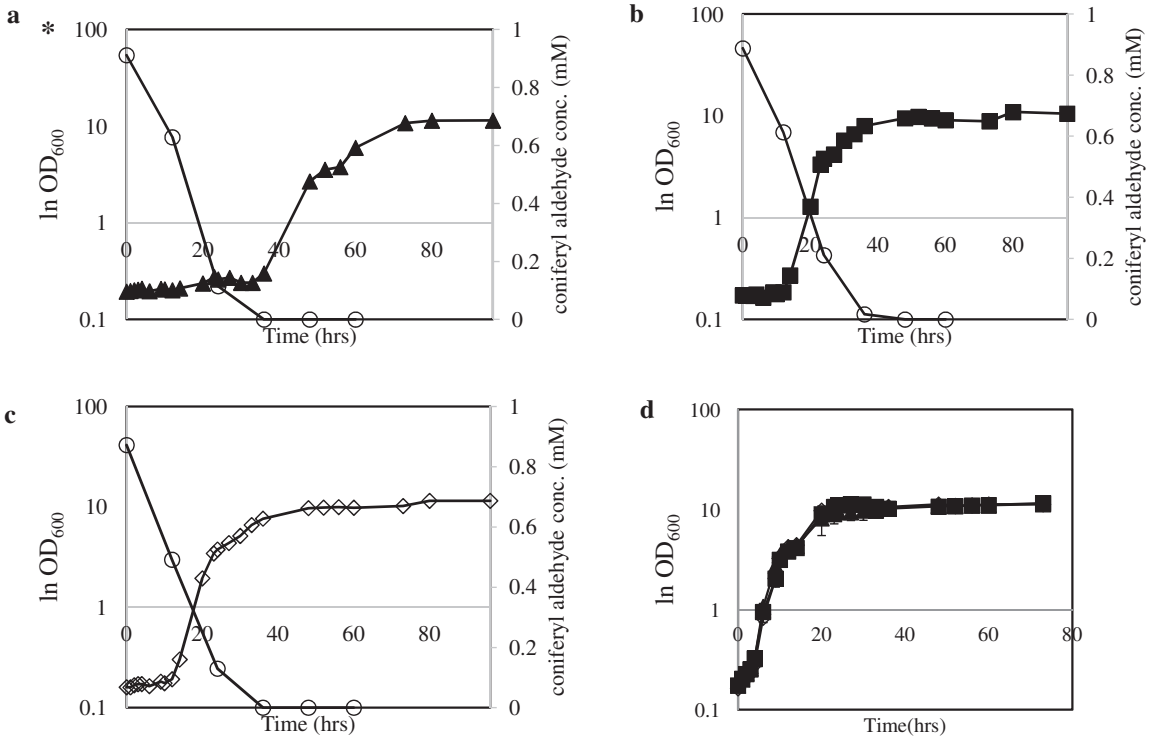

Fig. 4. Effect of the conversion of coniferyl aldehyde on the lag phase of recombinant strains (a) *B_CALD* (), (b) *SC_ald5Δ* (), (c) the control strain () and (d) *B_CALD*, *SC_ald5Δ* and the control strains in yeast minimal mineral medium without coniferyl aldehyde. Asterisks denoting significant differences between engineered strains and the control strain are shown when applicable.

Table 3
Average volumetric rate of conversion of coniferyl aldehyde in the first 12 and 24 h of conversion and the specific conversion rate of coniferyl aldehyde by *B_CALD*, *SC_ald5Δ* and the control strains. Higher rates are indicated with asterisks for the *B_CALD* strain. Asterisks denotes a significant difference between the *B_CALD* and other strains.

Strain	Volumetric conversion rate (mM/h)		Specific conversion rate of coniferyl aldehyde (g/g/h)
	0–12 h	0–24 h	
B_CALD	0.0240 ± 0.0003	0.0330 ± 0.0004*	0.030 + 0.003*
SC_ald5Δ	0.0230 ± 0.0003	0.0280 ± 0.0004	0.0009 ± 0.0001
Control	0.0320 ± 0.0004	0.0310 ± 0.0004	0.0011 ± 0.0003

control were 0.024 ± 0.0003 mM/h, 0.023 + 0.0003 mM/h and 0.032 ± 0.0004 mM/h respectively. After 24 h of cultivation, the volumetric conversion rate increased to 0.033 ± 0.0004 mM/h and 0.028 ± 0.0004 mM/h in the *B_CALD* and the deletion *SC_ald5Δ* strains, respectively, while the control strain maintained a steady conversion volumetric conversion rate of 0.031 ± 0.0004 mM/h, as it could be expected due to the delayed growth of *B_CALD*. The specific conversion rate over a period of 36 h showed the *B_CALD* strain converted coniferyl aldehyde at 0.030 ± 0.003 g/g/h of cells while the *SC_ald5Δ* converted at 0.0009 ± 0.0001 g/g/h and the control strain at 0.0011 ± 0.0003 g/g/h (Table 3). The *B_CALD* strain thus exhibited a specific conversion rate that is 33 times and 27 times higher than the *SC_ald5Δ* strain the control, respectively.

Although the *SC_ald5Δ* strain was still able to convert coniferyl aldehyde, this strain showed the slowest specific conversion rate. This supports the hypothesis that Ald5 is actively involved with the conversion of coniferyl aldehyde. The aldehyde dehydrogenase family in *S. cerevisiae* consists of 5 members that have been characterized and sequentially named *ALD2-ALD6*. *ALD2* (YMR170c), *ALD3* (YMR169c) and *ALD6* (YPL061w) are cytosolic, while *ALD4* (YOR374w) and *ALD5* (YER073w) are mitochondrial (Saint-Prix et al., 2004). *ALD* genes have been reported to exhibit redundancy, although they use different co-factors (Boubekeur et al., 1999; Saint-Prix et al., 2004). The redundancy in the *ALD* gene family may explain why the *SC_ald5Δ* strain was still capable of converting coniferyl aldehyde, even though it exhibited the highest sensitivity to 1.1 mM coniferyl aldehyde in the tolerance test and the conversion rate was lower. Also, the *SC_ald5Δ* strain had acetate yields slightly lower than that of the control strain. In literature, *ALD5* has been reported to be involved in regulation or biosynthesis of electron transport chain components and acetate formation via oxidation of acetaldehyde produced from pyruvate during the fermentation of sugars and that formed during ethanol oxidation (Saint-Prix et al., 2004; Walkey et al., 2012). However, *ALD5* has been shown to facilitate acetate formation under anaerobic growth conditions (Saint-Prix et al., 2004; Walkey et al., 2012). In this study, all cultivations have been done under aerobic condition, this could explain why acetate formation was not significantly different between the *SC_ald5Δ* and the control strains.

Conclusively, the *B_CALD* strain exhibited efficient conversion of coniferyl aldehyde, a trait which is valuable when developing microorganisms that are both robust and useful for a more efficient utilization of substrates rich in phenolic inhibitors as well as production of specific metabolites. In the concept of biorefinery, where complex natural substrates are used, a strain that has the potential for bioethanol and biochemical production is vital. Although this performance might have come at the expense of cell growth within the first 36 h, the strain however rapidly recovered at the end of the conversion.

4. Conclusion

This study was aimed at developing a *S. cerevisiae* strain with an improved ability to convert coniferyl aldehyde and investigate whether the strain would exhibit increased tolerance to coniferyl aldehyde. Efforts were also made to identify an enzyme involved in coniferyl aldehyde conversion in *S. cerevisiae*.

A successful attempt towards developing a strain of *S. cerevisiae* strain with improved endogenous conversion of phenolic compounds by heterologous expression of a known coniferyl aldehyde dehydrogenase enzyme from *Pseudomonas* was demonstrated. The performance of *SC_ald5Δ* strain strongly indicated that Ald5 is involved with the conversion of coniferyl aldehyde in *S. cerevisiae*.

Authors' contributions

Adeboye, P.T. designed and performed the experiments and wrote the article. Bettiga, M. and Olsson, L. reviewed the experimental design, data and the manuscript.

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Paper IV

Adeboye PT, Bettiga M, Olsson L. *ALD5*, *PAD1*, *ATF1* and *ATF2* facilitate the catabolism of coniferyl aldehyde, ferulic acid and *p*-coumaric acid in *Saccharomyces cerevisiae.* Submitted for publication.