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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:

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

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

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

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:

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

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

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

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

$$q_{product} = q_{substrate} * Y_{p/s} \quad (5)$$

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	–

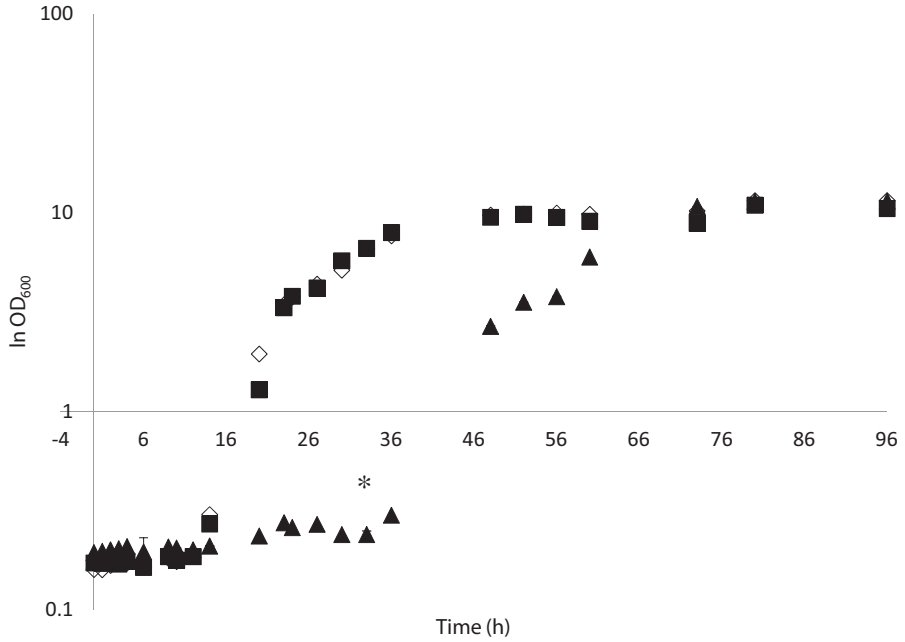

Fig. 1. Cell growth, expressed as the optical density in terms of the adsorption at 600 nm, of the investigated strains; *B_CALD* (▲), *SC_ald5Δ* (■), and the control strain (◆) in medium containing 1.1 mM coniferyl aldehyde. Asterisk denotes a significant difference between the *B_CALD* strain and other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

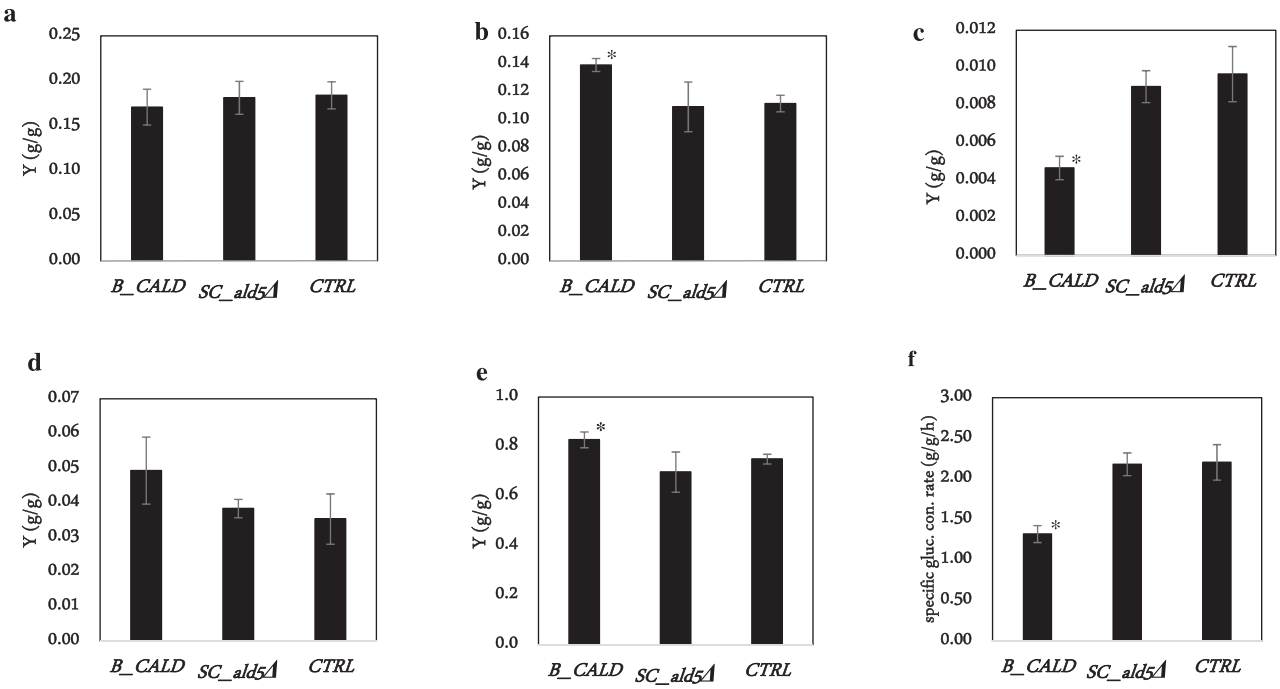

Fig. 2. Effect of 1.1 mM coniferyl aldehyde on the yields of (a) ethanol, (b) biomass, (c) acetate, (d) glycerol and (e) CO_2 from the cultivations of recombinant strains *B_CALD*, *SC_ald5Δ* and control using glucose as carbon source. (f) The specific glucose consumption rate. Asterisks denote differences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

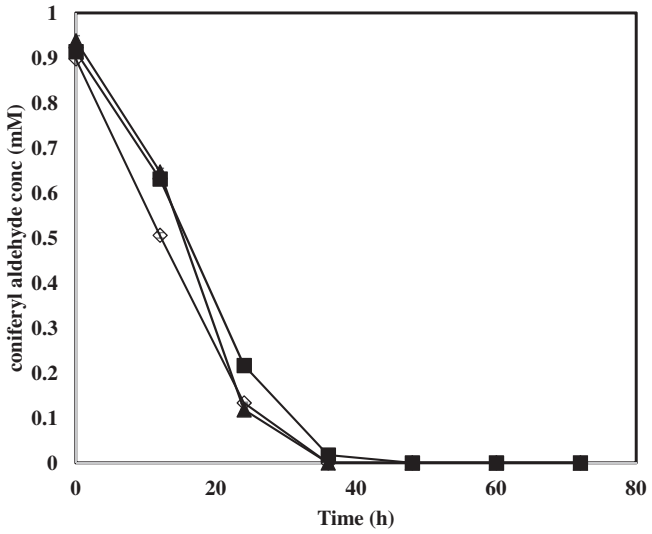

Fig. 3. Conversion of coniferyl aldehyde by the engineered strains *B_CALD* (▲), *SC_ald5Δ* (■), and the control strain (◆).

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

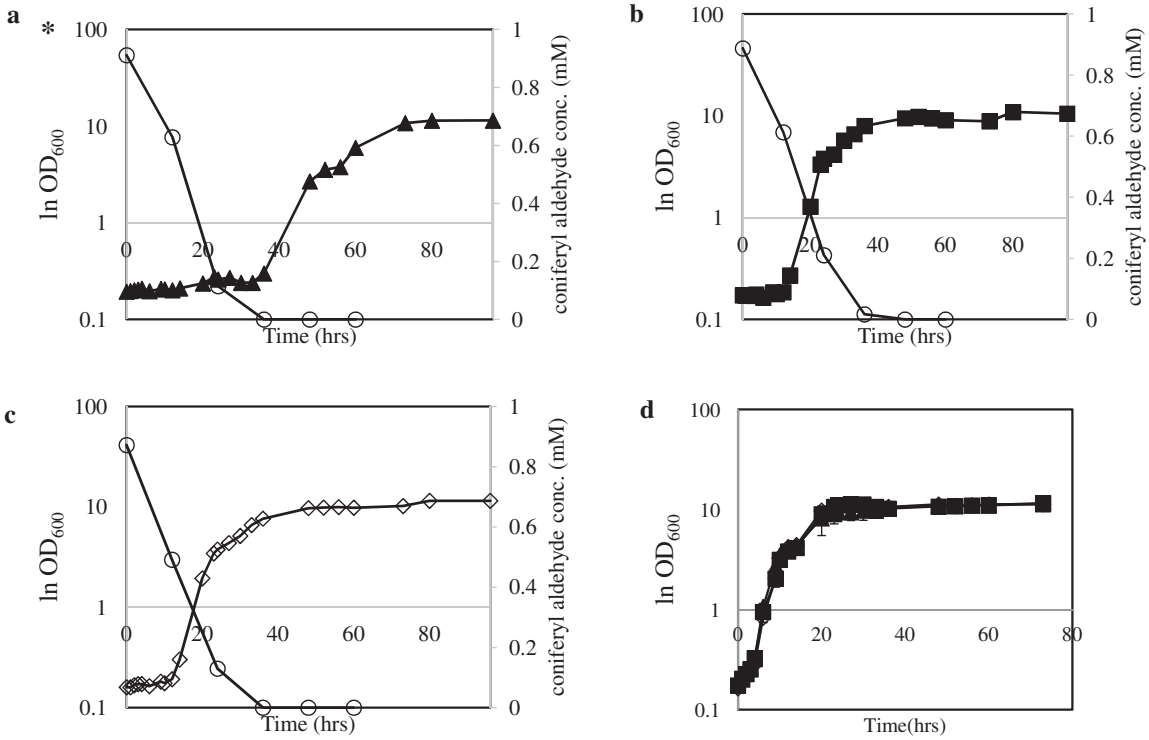

Fig. 4. Effect of the conversion of coniferyl aldehyde on the lag phase of recombinant strains (a) *B_CALD* (▲), (b) *SC_ald5Δ* (■), (c) the control strain (◆) and (d) *B_CALD*, *SC_ald5Δ* and the control strains in yeast minimal mineral medium without coniferyl aldehyde. Asterisks denoting significant 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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