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Organic acids from lignocellulose: *Candida lignohabitans* as a new microbial cell factory

Martina Bellasio · Diethard Mattanovich · Michael Sauer · Hans Marx

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Abstract Biorefinery applications require microbial cell factories for the conversion of various sugars derived from lignocellulosic material into value-added chemicals. Here, the capabilities of the yeast *Candida lignohabitans* to utilize a range of such sugars is characterized. Substrates efficiently converted by this yeast include the pentoses xylose and arabinose. Genetic engineering of *C. lignohabitans* with the isolated endogenous GAP promoter and GAP terminator was successful. GFP expression was used as a proof of functionality for the isolated transcription elements. Expression of lactate dehydrogenase and cis-aconitate decarboxylase resulted in stable and reproducible production of lactic acid and itaconic acid, respectively. The desired organic acids were accumulated converting pure sugars as well as lignocellulosic hydrolysates. *C. lignohabitans* proved therefore to be a promising reliable microbial host for production of organic acids from lignocellulosic material.

Keywords Microbial cell factory · *Candida lignohabitans* · Lactic acid production · Itaconic acid production · Biorefinery

M. Bellasio · D. Mattanovich · M. Sauer (✉) · H. Marx
Department of Biotechnology, BOKU—VIBT University of Natural Resources and Life Sciences, Muthgasse 107, 1190 Vienna, Austria
e-mail: michael.sauer@boku.ac.at

D. Mattanovich · M. Sauer
Austrian Centre of Industrial Biotechnology (ACIB GmbH), Vienna, Austria

Introduction

Lignocellulosic biomass is the most relevant renewable resource for material production. It is obtainable at low costs and at large scale from agriculture and forestry, potentially enabling environmentally friendly production processes, noncompetitive with the food chain.

Great emphasis is therefore placed onto the biotechnological utilization of lignocellulosic biomass. In this sense, the complete conversion of the cellulose and hemicellulose contained in the material represents a key challenge toward the setup of sustainable second-generation production processes.

To obtain the sugars contained in these polymers, pretreatment of the lignocellulosic biomass is inevitable [6]. The pretreatment of choice will disrupt the tight structure of lignocellulosic material, leaving areas more accessible to cellulolytic and hemicellulolytic enzymes. From the hydrolysis of cellulose only glucose will be obtained, while from the hydrolysis of hemicellulose various sugars, like xylose, arabinose or mannose will be released. However, the harsh conditions required for the pretreatment will also lead to the production of toxic degradation products (i.e., furfural, hydroxymethylfurfural, phenolic compounds, organic acids).

The necessary consumption of hexose and pentose sugars, together with the concomitant presence of various inhibitors, makes lignocellulosic hydrolysates challenging substrates for the growth of many microorganisms. Extensive research has been performed in recent years to engineer the well-known biotechnological platform organism *Saccharomyces cerevisiae* for the utilization of xylose and arabinose [2, 3, 8] and for increased resistance to inhibitors [27, 35].

Another approach for conversion of lignocellulosic material is represented by microorganisms naturally able

to consume pentose sugars. A large number of yeasts have shown the ability to metabolize xylose and arabinose, but only very few are reported to be able to ferment xylose to ethanol [9, 31]. Although bioethanol remains the main focus of lignocellulosic biomass conversion, increasing interest is directed toward the conversion of lignocellulosic material into other bulk chemicals, such as building blocks for the production of biopolymers.

Comprehensive research is being conducted to find suitable microbial cell factories that can directly grow on non-detoxified lignocellulosic hydrolysates, converting the sugars into the value-added chemical of interest.

Here, we present *Candida lignohabitans* (also described as *Sugiyamaella lignohabitans* [32]), a yeast strain belonging to the *Sugiyamaella* clade [15, 16] as a novel microbial cell factory for biorefinery applications. As examples of products of interest, itaconic acid and lactic acid were chosen. Applications of itaconic acid (2-methylidenebutanedioic acid) and its derivatives range from co-monomer to prepare acrylic fibers and rubbers to binder in non-woven fibers, paper and concrete paint. More recently, applications of itaconic acid were found in pharmacy, dentistry and optometry [21]. Lactic acid is a carboxylic acid widely used as building block for polylactide polymers (polylactic acid, PLA), a biodegradable and environmentally friendly plastic suitable as substitute for petrochemical plastics [28]. Extensive research has been done to set up a cost-effective production and downstream process for lactic acid [23, 24, 28]. The utilization of cheap substrates such as lignocellulosic biomass represents therefore an attractive option for the economically sustainable production of itaconic acid and lactic acid.

Materials and methods

Strains and media

The strain *C. lignohabitans* CBS 10342 was obtained from the CBS-KNAW Fungal Biodiversity Centre, The Netherlands. Cells were maintained at −80 °C in YPD medium (1 % yeast extract, 2 % soy peptone, 2 % glucose) supplemented with 10 % (w/v) glycerol. Yeast cultures were performed in standard 500 ml Schott Duran shake flask filled with 30 ml medium, at 30 °C, 180 rpm, 25 mm shaking diameter. Non-buffered YP medium (1 % yeast extract, 2 % soy peptone) was supplemented with 2 or 5 % pure carbon sources or with steam explosion treated, cellulase digested lignocellulosic material. In the text, YP supplemented with glucose is indicated as YPD, while YP supplemented with xylose is indicated as YPX. The pH of media containing hydrolysates was adjusted to 6.5 with addition of NaOH. Hygromycin B (150 µg/ml) or Zeocin (400 µg/ml) was added as selective agent when required. Yeast cultures in synthetic minimal medium contained YNB (Yeast Nitrogen Base w/o amino acids, Difco) with 2 % carbon sources.

To prepare agar plates containing a pH gradient, plates (Square BioAssay Dish, 245 mm × 245 mm, Corning®) were kept elevated on one side and half-filled with YPD-agar pH 9. After solidification, plates were laid down and YPD-agar pH 1.5 was added. The plates were incubated for 24 h to allow the formation of a linear gradient prior to streaking the strains. Overnight YPD cultures of selected strains were harvested by centrifugation, washed once with sterile water and diluted to an OD_{600} of 0.005. 50 µl of the diluted culture was placed at the center of the plate and streaked across the pH gradient with a sterile loop. Plates were incubated for 48 h at 30 °C.

Preparation of lignocellulosic hydrolysates

Straw, Miscanthus, sawdust, shrub cuttings and wood chips containing 30 % (w/w) spruce and 70 % (w/w) beech were chosen as lignocellulosic biomass. A steam explosion treatment (15 min at 121 °C, 2 bar) was performed and the samples were stored at −20 °C until further treatment. The dry matter content of the lignocellulosic samples was determined and adjusted to 20 % with water. Cellic Ctec2® (Novozymes®, USA) was added according to the manufacturer's recommendations and the enzymatic digestions were performed in shake flasks at 50 °C, 220 rpm, for 72 h. The liquid fraction resulting from the digestion was separated from the solid residues by centrifugation, filter-sterilized and used as stock for preparation of media.

Molecular biology techniques

Escherichia coli DH10B was used as host for DNA manipulation. *E. coli* transformants were grown on LB [25] supplemented with 25 µg/ml Zeocin or 100 µg/ml hygromycin B.

C. lignohabitans was transformed by an electroporation method. In brief, an overnight culture was used to inoculate a 50 ml YPD culture to an OD_{600} of 0.5. After the culture reached an OD_{600} of 2, cells were collected by centrifugation, resuspended in 25 ml YPD containing 0.5 ml of 1 M HEPES and 0.625 ml of 1 M DTT, and incubated for 15 min at 30 °C, 180 rpm. Cells were washed with ice-cold 1 mM HEPES and subsequently resuspended in 250 µl of 1 M sorbitol. 5–10 µg of linearized DNA was added to 80 µl of the competent cells' suspension and the mixture was transferred into a chilled electroporation cuvette. Electroporation was performed using the Biorad Gene Pulser Xcell Electroporation System at 1,500 V, 25 µF, 200 Ω.

Cells were allowed to regenerate for 3 h in YPD. Transformants were selected on YPD plates supplemented with hygromycin B (150 µg/ml) or Zeocin (400 µg/ml). Up to 10 transformants/µg DNA were obtained.

Genomic DNA of *C. lignohabitans* was prepared using the QIAGEN Genomic-Tip 100/G kit according to the manufacturer's instruction manual.

The isolation of the endogenous GAP promoter and terminator followed an inverse PCR protocol. The genomic DNA was digested with a restriction enzyme (*Eco*RI, *Spe*I and *Xho*I were used). After heat inactivation of the restriction enzyme, intramolecular circularization of the obtained fragments was allowed by adding T4 ligase. The DNA was cleaned following an ethanol precipitation protocol and used as template for PCR.

Primers were designed assuming that the coding sequence for a critical enzyme such as the glyceraldehyde 3-phosphate dehydrogenase would be conserved between yeasts belonging to the same class. Therefore, an alignment of the GAP ORF from different Saccharomycetes (i.e., *S. cerevisiae*, *Candida albicans*, *Pichia pastoris*) was created, and the primers GAPorf_fw and GAPorf_bw (Table 1) were designed on the most conserved regions of the coding sequence. A PCR (2 min 98 °C, followed by 35 cycles; each cycle consisted of 10 s at 98 °C, 30 s at 44 °C and 30 s at 72 °C) using the library of circularized genomic DNA as template and GAPorf_fw and GAPorf_bw as primers resulted in a 550-bp fragment. The fragment was subcloned in the vector pSTBlue-1 utilizing the Perfectly Blunt Cloning Kit (Novagene) and sequenced. This sequenced part of the endogenous GAP ORF was used to design specific primers for an inverse PCR. The primers GAPdownstream_fw and GAPupstream_bw (Table 1) were used to perform a PCR using the circularized genomic DNA as template (2 min 98 °C, followed by 35 cycles; each cycle consisted of 10 s at 98 °C, 30 s at 68 °C and 60 s at 72 °C). Several of the obtained bands were gel-purified, subcloned in the vector pSTBlue and sent for sequencing. The sequence of the whole GAP gene was reconstructed: a region of 800 bp upstream to the GAP ORF was identified as the GAP promoter (pGAP) and a region of 200 bp downstream was identified as the GAP terminator (GAPtt).

Table 1 Primers used for the isolation of pGAP and GAPtt

GAPorf_fw	5′ TATGCTGCTTACATGTTCAA 3′
GAPorf_bw	5′ GTTCTACCACCTCTCCAGT 3′
GAPdownstream_fw	5′ ACGCTTACAAGCCTGAGTACAAGGTC 3′
GAPupstream_bw	5′ CAACAACGTAGTCAGCACCGG 3′

The L-LDH coding sequence (GenBank X70926) was PCR-amplified from the genomic DNA of *Lactobacillus plantarum* ATCC 8014. The CAD1 coding sequence (accession number: B3IUN8.1) was PCR-amplified from the vector pCAD_*mbfA* [4]. The enhanced green fluorescent protein coding sequence (eGFP) was PCR-amplified from pET30a_GFP-mut3.1 [30]. The final vectors used for transformation of *C. lignohabitans* contained expression cassettes as described in the text, pUC19 from pGAPZ B (Invitrogen), NTS region amplified from *P. pastoris* X-33 genomic DNA, Zeocin resistance cassette from pGAPZ B (Invitrogen) or hygromycin B resistance cassette. Hygromycin B resistance cassette was constructed using hygromycin B phosphotransferase coding sequence [7], under the control of TEF promoter from *S. cerevisiae* (pGAPZ B, Invitrogen) and CYC terminator from *S. cerevisiae* (pGAPZ B, Invitrogen). The constructed plasmids were linearized at the NTS region level with SpeI (New England Biolabs) before transformation.

The expression vectors for *C. lignohabitans* were constructed following a modular cloning system [33]. Multiple DNA fragments were assembled in a one-pot reaction in presence of a type IIS restriction enzyme (*Bbs*I or *Bsa*I, New England Biolabs), T4 ligase (New England Biolabs) and T4 ligase buffer. The assembly was incubated allowing 45 cycles of restriction and ligation (respectively, 37 °C, 1 min and 16 °C, 2.5 min), and transformed into *E. coli* DH10B.

Fluorescence measurements

Overnight cultures were harvested by centrifugation and resuspended in water to a final OD_{600} of 0.5. Fluorescence quantifications were performed in 96-well plates (Fluoro-Nunc; Nunc) on a fluorescence photometer (Infinite M200 Tecan plate reader), with excitation wavelength at 488 nm and emission wavelength at 530 nm.

Pictures of fluorescent cells were taken with a Leica DMI 6000 fluorescence microscope equipped with an HCX PL Apo 100 × 1.40 NA oil immersion objective and a Leica CCD camera. Brightness and contrast were adjusted in ImageJ.

Biomass quantification

Biomass was quantified by measuring the optical density of culture samples at 600 nm. A correlation between OD_{600} and dry biomass content was established. Interestingly, the correlation is: $OD_{600}/5.546$ = g/l dry biomass during the initial exponential growth phase on sugars, but decreases to $OD_{600}/9.703$ = g/l dry biomass during the later growth phase on fermentation products (no significant change in morphology can be observed).

HPLC measurements

The concentrations of D-glucose, D-xylose, D-mannose, D-galactose, L-arabinose, lactic acid, glycerol, ethanol, acetic acid and itaconic acid in the culture were determined by HPLC analysis (Shimadzu, Korneuburg, Austria). Samples were prepared for the analysis adding H_2SO_4 to a final concentration of 4 mM; the samples were filtrated and 10 µL were injected in a Rezex ROA-Organic Acid H + column (300 × 7.8 mm, Phenomenex, USA). The column was operated with 4 mM H_2SO_4 as mobile phase, with 1.0 ml/min flow rate at 60 °C. A refraction index detector (RID-10A, Shimadzu, Korneuburg, Austria) was used for quantification of sugars, when used separately, lactic acid, glycerol, ethanol and acetic acid. 0.1 g/l of each compound was established as reliable quantification limit with this method. A photodiode array detector (SPD-M20A, Shimadzu, Korneuburg, Austria) was used for quantification of itaconic acid at 254 nm.

For the quantification of D-glucose, D-xylose, D-mannose, D-galactose, L-arabinose, furfural and hydroxymethylfurfural in steam explosion hydrolysates (mixture of sugars), samples were diluted with water if necessary, filtrated and 10 µl was injected in a Rezex RPM-Monosaccharide Pb +2 (8 %) column (300 × 7.8 mm, Phenomenex, USA). The column was operated with H_2O as mobile phase, with 0.6 ml/min flow rate at 80 °C. Detection with RID as above.

Results and discussion

Growth on different carbon sources

The metabolic capabilities of *C. lignohabitans* were first evaluated in aerated liquid cultures supplemented with different carbon sources. Several hexoses (glucose, mannose and galactose) and pentoses (xylose and arabinose) were chosen, which are the major components of cellulose and hemicellulose, respectively.

The ability of the strain to consume different carbon sources was tested both in complex and minimal medium (Table 2). In each of the conditions tested, the carbon source was entirely consumed. As a result, a high amount of biomass was accumulated during the depletion of each sugar. The growth curves in YP medium for a representative replicate are shown in Fig. 1. The biomass yield was the same for all hexoses and pentoses.

The production of metabolites during these aerated liquid cultures was also monitored at different time points. Ethanol (Table 2) was detected in all the cultures in complex medium, with the highest ethanol yield (0.2 g/g, 4 g/l) obtained after 22 h of growth, when a complete depletion of glucose had occurred. A maximum of 1 g/l of ethanol was instead accumulated in 24 h after consumption of 20 g/l xylose (0.05 g/g). Ethanol was also detectable during the aerated growth on minimal medium supplemented with glucose and xylose. *C. lignohabitans* shows here the remarkable capability to ferment both pentoses and hexoses. Many yeast strains have indeed shown the capability to consume xylose and arabinose, but only very few are reported to convert pentoses into ethanol [9].

Ethanol was not detected when minimal medium was supplemented with mannose, galactose and arabinose. This discrepancy between what was observed on rich and minimal medium is probably due to the lower amount of biomass accumulated by the strain on minimal medium.

C. lignohabitans also showed to accumulate significant amounts of glycerol in rich medium. A maximum of 2 g/l were obtained after the consumption of 20 g/l glucose, and 1.8 g/l were obtained from 20 g/l xylose. Glycerol is a known by-product of fermentation of sugars to ethanol, contributing to the maintenance of the redox balance in

Table 2 Assimilation of hexoses and pentoses and production of ethanol on rich and defined medium by the yeast *C. lignohabitans*

	Growth		Ethanol production	
Carbon source	YP+	YNB+	YP+	YNB+
Glucose	+	+	+	+
Xylose	+	+	+	+
Arabinose	+	+	+	n.d.
Galactose	+	+	+	n.d.
Mannose	+	+	+	n.d.

n.d. the ethanol concentration was below the detection limit

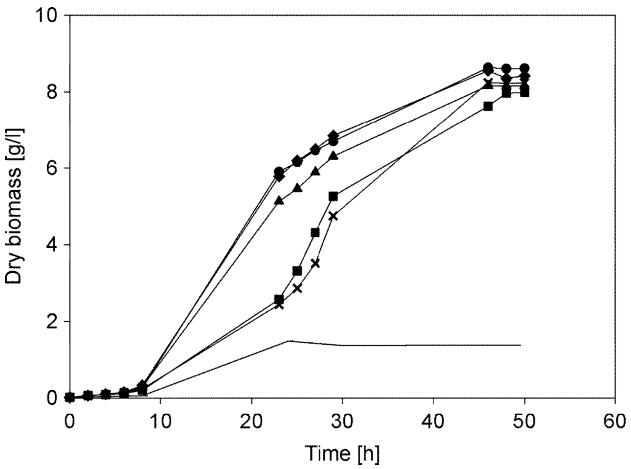

Fig. 1 A representative replicate of *C. lignohabitans* growth in shake flasks at 30 °C on YP medium supplemented with no sugars (*straight line*), 2 % glucose (*filled circles*), 2 % xylose (*filled triangles*), 2 % mannose (*filled diamonds*), 2 % galactose (*filled squares*) and 2 % arabinose (*crosses*)

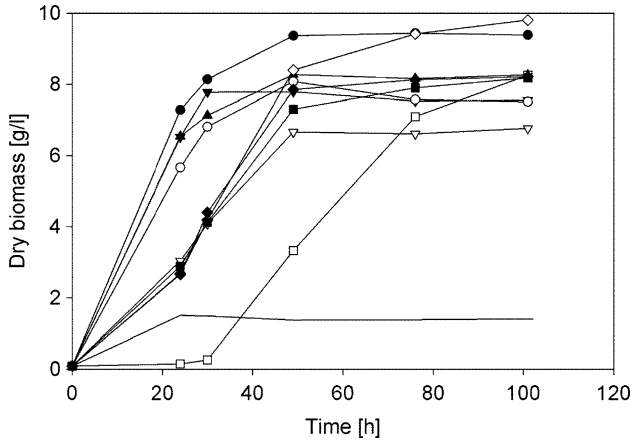

Fig. 2 Growth of *C. lignohabitans* in shake flasks at 30 °C on YP medium supplemented with no sugars (*straight line*) 2 % glucose (*filled circles*), 2 % xylose (*filled triangles*), 2 % sugars mix as in wood chips' hydrolysates (*filled inverted triangles*), and lignocellulosic hydrolysates, with 2 % total sugar content (straw, *open triangles*; Miscanthus, *open squares*; sawdust, *open diamonds*; Beech wood chips, *filled diamonds*; Chopped wood chips, *open inverted triangles*; Shrub cuttings, *filled squares*)

the cell in oxygen-limited cultures. Here, the experimental setup in shake flasks, together with the high biomass concentration reached in these conditions, likely creates a microaerobic environment, which may trigger the production of both ethanol and glycerol.

To further characterize the range of carbon sources that can be consumed by *C. lignohabitans*, liquid cultures were supplemented with other substrates. In rich medium, the strain was able to consume ethanol, lactate, glycerol and acetate, but not itaconate. A large range of possible carbon sources was therefore defined for *C. lignohabitans*, showing the versatility of this strain for a wide range of biorefinery applications.

The ability of *C. lignohabitans* to grow on rich medium supplemented with hydrolyzed lignocellulosic material was investigated. A variety of lignocellulosic sources was chosen, to resemble a broad spectrum of feedstocks in a biorefinery process. The lignocellulosic hydrolysates were added to rich medium as the only sugar source, and the obtained growth curves are shown in Fig. 2. After 72 h, the strain had accumulated comparable amounts of biomass in each of the conditions tested. Some inhibition was shown only in the first phases of the growth, particularly in presence of straw and shrub hydrolysates. Furfural was present in straw and shrub hydrolysates with concentrations of 0.22 and 0.17 g/l, respectively, while it was not detectable in the other hydrolysates. It is therefore tempting to correlate the elongated lag phase with the presence of furfural, which has previously been indicated as one of the major toxicity components in lignocellulosic hydrolysates [11], acting as inhibitor of enzymes involved in the central carbon metabolism [19]. Furthermore, as already reported for other yeasts [11, 18] during the lag phase a conversion of furfural into the less toxic furfuryl alcohol was observed (data not shown), enabling the strain to start the consumption of the carbon sources in the medium.

pH tolerance

Acid tolerance is a requirement for a microbial cell factory in biorefinery applications. While some pretreatments of lignocellulosic biomass imply the usage of acidic conditions [6], the hydrolysis of hemicellulose liberates acetic acid, which also results in a decreased pH. Moreover, many important target compounds for biorefineries are organic acids, whose accumulation will also lower the pH and decrease the vitality of the host itself.

A test for pH tolerance was therefore conducted: *C. lignohabitans* was compared to other yeasts (i.e., *S. cerevisiae*, *Kluyveromyces marxianus*, *P. pastoris*, *Candida shehatae*, *Candida lignosa*) in terms of growth on a YPD plate containing a pH gradient from 1.5 to 9 (Fig. 3a). After 48 h of incubation, growth in the most acidic area of the plate can be observed only for *C. lignohabitans*. The remarkable pH tolerance of *C. lignohabitans* was also confirmed in liquid cultures with rich medium (Fig. 3b). Here, MES buffer was chosen as it does not represent an additional carbon source to the culture. The strain exhibits a comparable behavior at pH 6 and 3. When the initial pH was set to 2.5, MES buffer was not able to prevent a pH drop to 2 during the first 20 h of growth; however, *C. lignohabitans* accumulated a considerable amount of biomass also in this condition.

As previously mentioned, a biorefinery process requires a microorganism to be tolerant not only to low pH, but also the presence of organic acids at low pH. The acids in their undissociated form will permeate into the cells, where they will dissociate due to the higher intracellular pH. The necessity to maintain the intracellular pH homeostasis will determine high energy consumption, ultimately causing decreased cell viability.

To start investigating on *C. lignohabitans* tolerance to organic acids at low pH, aerated cultivations in presence of various concentrations of lactic acid at pH 3.5 were performed (Fig. 3c). At this pH most of the lactic acid (pK_a 3.86) is in its protonated form. The experimental data show how the increasing lactic acid concentration affects the growth; however, the strain was able to exhibit a significant biomass accumulation in presence of a concentration of lactic acid as high as 30 g/l. The response of yeast cells to organic acids can anyway be variable, depending on the strain used, the organic acids concentration and the process conditions [28]. Different organic acids can also cause very

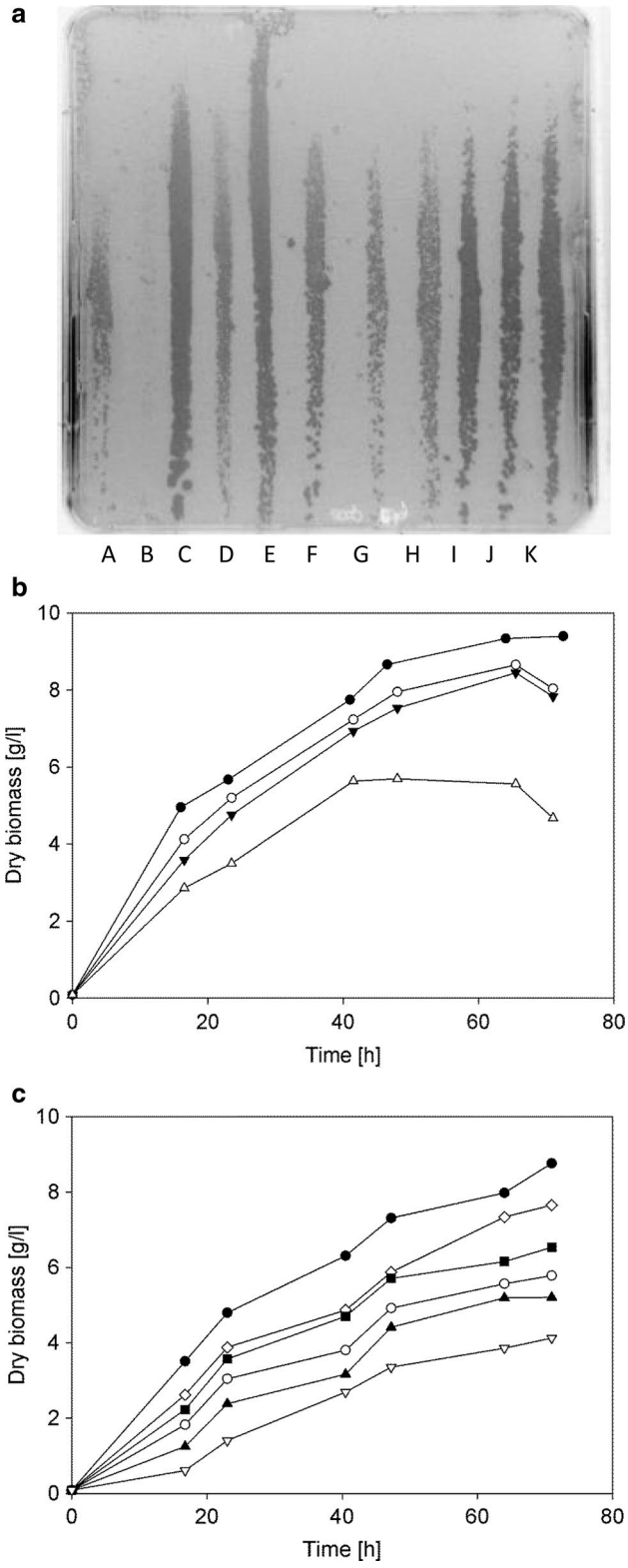

Fig. 3 **a** YPD plate containing a pH gradient from 1.5 (*top*) to 9.0 (*bottom*). *A*: *P. pastoris* X-33; *B*: *C. shehatae* CBS 4410; *C*: *K. marxianus* CBS 6397; *D*: *C. lignosa* CBS 4705; *E*: *C. lignohabitans* CBS 10342; *F*: *S. cerevisiae* CEN PK; *G*: *S.cerevisiae* CBS 429; *H*: *S. cerevisiae* CBS 6006; *I*: *S.cerevisiae* CBS 7960; *J*: *S. cerevisiae* CBS 7961; *K*: *S. cerevisiae* CBS 7962. **b** Growth curves of *C. lignohabitans* in YPD at pH 6 (*filled circles*), pH 3.5 (*open circles*), pH 3 (*filled inverted triangles*), pH 2.5 (*open triangles*). **c** Growth curves of *C. lignohabitans* in YPD, pH 3.5, supplemented with lactic acid at a final concentration of 5 g/l (*filled circles*), 10 g/l (*open diamonds*), 15 g/l (*filled squares*), 20 g/l (*open circles*), 25 g/l (*filled triangles*), 30 g/l (*open inverted triangles*)

different responses. The presence of 5 g/l of itaconic acid at pH 3.5, significantly inhibit the growth of *C. lignohabitans* in aerated shake flask cultures (data not shown).

Development of genetic tools for *C. lignohabitans* manipulation

The metabolic capabilities highlighted here make *C. lignohabitans* an interesting potential microbial cell factory to convert lignocellulosic biomass into valuable chemical building blocks. However, genetic manipulation of *C. lignohabitans* has not been described in the literature before. A first attempt for transformation of the strain was performed with a standard electroporation method, using a linearized hygromycin B resistance cassette, containing the TEF promoter from *S. cerevisiae*. A number of clones were obtained, yielding a transformation efficiency of 10 colonies per µg of DNA. All obtained transformants showed to stably retain the acquired antibiotic resistance, probably because of integration of the transformed DNA into the genome of *C. lignohabitans*.

One question to address when aiming at the transformation of *Candida* strains is whether the strain belongs to the CTG clade or not. The majority of species belonging to the *Candida* genus use a peculiar codon usage [13, 22, 26]. *C. albicans*, *C. tropicalis*, *C. famata* and *C. guilliermondii*—to name some examples—translate the codon CTG as serine instead of leucine and are therefore referred to as the CTG clade. Since the codon usage is fundamental for the correct translation of any expressed sequence, a simple transformation test was performed to establish whether *C. lignohabitans* also belonged to the CTG clade.

The HPH gene codes for hygromycin phosphotransferase, an enzyme that confers resistance to hygromycin; its coding sequence contains nine CTG codons, which have been substituted with nine CTC codons by site-directed mutagenesis by Basso et al. [1]. It has been demonstrated that the non-codon-optimized HPH gene does not confer any resistance to hygromycin in yeasts belonging to the CTG clade [10], while codon-optimized HPH ORFs have demonstrated to be effective in conferring resistance to hygromycin in *Candida* [1, 34]. On the other hand, both non- and codon-optimized HPH ORFs can confer hygromycin resistance to a yeast using a standard genetic code, since both the codons CTG and CTC would in this case be translated as leucine.

When *C. lignohabitans* was transformed with a standard HPH resistance cassette and with a cassette containing the codon-optimized HPH, both cassettes conferred resistance to hygromycin (not shown), leading to the conclusion that *C. lignohabitans* is not part of the CTG clade. Therefore, all the subsequent metabolic engineering work was performed without any substitution of CTG codons. Interestingly, these data also indicate that both the promoters present in the cassettes used (i.e., TEF promoter from *S. cerevisiae* and TEF promoter from *C. albicans*) can trigger the transcription in *C. lignohabitans*.

Identification of GAP promoter and GAP terminator

To allow an efficient and stable expression of genes of interest, we decided not to rely on the TEF promoters contained in the previously used hygromycin resistance cassettes, but to isolate an endogenous, strong, constitutive promoter. The GAP (glyceraldehyde 3-phosphate dehydrogenase) promoter is commonly used in other expression systems, e.g., for *P. pastoris* and *H. polymorpha*. The isolation of the GAP promoter of *C. lignohabitans* was therefore an important step to obtain stable, strong expression of a gene of interest. With an inverse PCR amplification of the regions upstream and downstream the GAP ORF, the GAP promoter (pGAP) and GAP terminator (GAPtt) of *C. lignohabitans* were obtained. The sequence of the whole GAP gene of *C. lignohabitans* is available in GenBank with the accession number KM371012.

The GAP ORF (gene name: *TDH1*) shows about 75 % nucleotide sequence identity with other *Saccharomycetes* like *Debaryomyces hansenii* and *C. albicans*. Interestingly, no significant similarities were found by comparing the sequences of GAP promoter or GAP terminator of *C. lignohabitans* in a nucleotide Basic Local Alignment Search Tool (BLAST).

To prove the functionality of the isolated pGAP and GAPtt, GFP was chosen as reporter gene. An expression cassette with the GFP ORF under the control of pGAP and GAPtt was constructed and transformed in *C. lignohabitans*. GFP expression was first verified by measuring the fluorescence emitted. Pictures of the transformants taken with a fluorescence microscope also show a strong cytosolic signal (Fig. 4), as a confirmation that GFP is correctly expressed at high levels in the transformed *C. lignohabitans* cells. pGAP and GAPtt were therefore confirmed as appropriate regulation sequences for the expression of genes in *C. lignohabitans*.

Itaconic acid production in *C. lignohabitans*

The enzyme cis-aconitate dehydrogenase (EC 4.1.1.6), encoded by the gene cadA, is responsible for the production of itaconic acid in *Aspergillus terreus*. Previous work demonstrated that it is possible to produce itaconic acid in different microorganisms by expressing the cadA gene [14]. The possibility of producing itaconic acid in *C. lignohabitans* was tested, constructing an expression cassette for cadA. The cadA coding sequence was expressed under the control of the isolated pGAP and GAPtt.

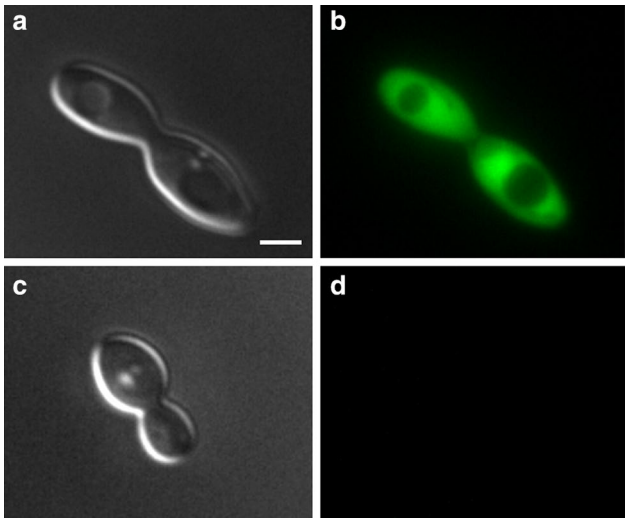

Fig. 4 *C. lignohabitans*-GFP cells with white light (**a**), UV light (**b**); *C. lignohabitans* wild-type cells with white light (**c**) and UV light (**d**). Bar: 1 µm

Different transformants were cultivated on YP supplemented with 50 g/l glucose. All the obtained clones showed a growth comparable with the wild-type strain, indicating that the performed genetic manipulation did not affect the growth capability of *C. lignohabitans*.

Each clone also produced itaconic acid, reaching a final concentration between 2 and 4 g/l. The concentration of glucose, ethanol, glycerol and itaconic acid was monitored during the growth and is shown in Fig. 5b for the best performing clone. Glucose is quickly consumed, leading to the production of ethanol (up to 10 g/l) and glycerol (up to 4 g/l) as fermentation products. Interestingly, only a little amount of itaconic acid is accumulated during glucose consumption. The concentration of itaconic acid increases instead up to 4 g/l during the depletion of ethanol. We hypothesize here the involvement of the glyoxylate cycle, which would enable the assimilation of ethanol through a conversion into citrate and isocitrate via aconitate. The reached concentration of itaconic acid (4 g/l) is remarkably higher than what has been previously observed with other recombinant microorganisms [14, 21].

The obtained transformants were subsequently cultivated on xylose, to verify the capability to produce itaconic acid from a pentose sugar. Also on this substrate all the clones showed a growth comparable with the wild-type

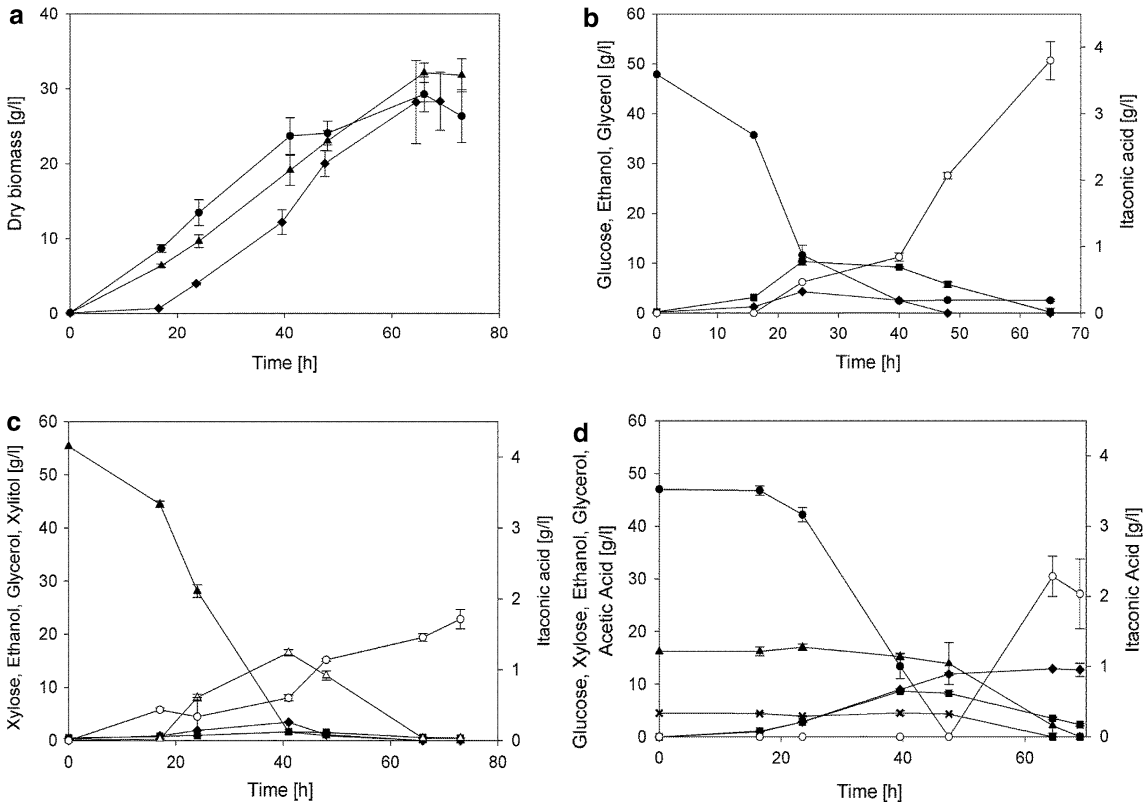

Fig. 5 **a** Growth curves of *C. lignohabitans*-CAD on YP medium supplemented with glucose 5 % (*filled circles*), xylose 5 % (*filled triangles*) and lignocellulosic hydrolysates (5 % total sugar content, *filled diamonds*). **b**, **c**, **d** Metabolite profiles during the growth of *C. lignohabitans*-CAD on YPD (**b**), YPX (**c**) and on YP supplemented with lignocellulosic hydrolysates (**d**). Glucose, *filled circles*; xylose, *filled triangles*; xylitol, *open triangles*; ethanol, *filled squares*; glycerol, *filled diamonds*; acetic acid, *crosses*; itaconic acid, *open circles*. (Shake flasks, 30 °C)

strain and accumulated itaconic acid (data not shown). In Fig. 5c, the trends of xylose, ethanol, glycerol, xylitol and itaconic acid are shown for one representative clone. During the consumption of xylose, accumulation of ethanol, xylitol and glycerol is observed. Also in this case, accumulation of itaconic acid is significantly higher during the depletion of ethanol and xylitol, rather than directly during the growth on xylose.

To finally test the ability to produce itaconic acid on lignocellulosic hydrolysate, an experiment using enzymatically digested wood chips as carbon source was performed. In this substrate, mainly glucose (47 g/l) and xylose (17 g/l) were present, while other sugars were not detectable. Acetic acid (4.5 g/l), furfural (0.43 g/l) and hydroxymethyl furfural (HMF; 0.09 g/l) were detected in the lignocellulosic hydrolysates. Growth kinetics of the recombinant *C. lignohabitans*-CAD strain on hydrolysates showed an extended lag phase, compared to the growth on pure glucose and xylose (Fig. 5a). However, after 60 h of growth the strain accumulated an amount of biomass comparable to the control on pure sugars.

The trends of the different metabolites during the growth of *C. lignohabitans*-CAD on lignocellulosic hydrolysates are shown in Fig. 5d. Glucose is consumed as the preferred carbon source, leading to accumulation of ethanol (8 g/l) and glycerol (10 g/l). After the depletion of glucose, other carbon sources, mainly xylose and ethanol, begin to be utilized. After 69 h of growth, the sugars contained in the hydrolysates were entirely consumed.

Also on this substrate, only in the late phases of growth accumulation of itaconic acid is observable. Up to 2.5 g/l of itaconic acid was produced, demonstrating the ability of the engineered *C. lignohabitans* to convert sugars from lignocellulosic hydrolysates into this value-added chemical.

Production of itaconic acid from lignocellulosic biomass represents an attractive alternative to the traditional production process. However, the natural itaconic acid producer *A. terreus* has shown significant sensitivity to inhibitory compounds contained in lignocellulosic hydrolysates [14]. Recombinant microorganisms represent therefore an attractive option. In this prospective, we present here a recombinant *C. lignohabitans* strain producing a considerably high

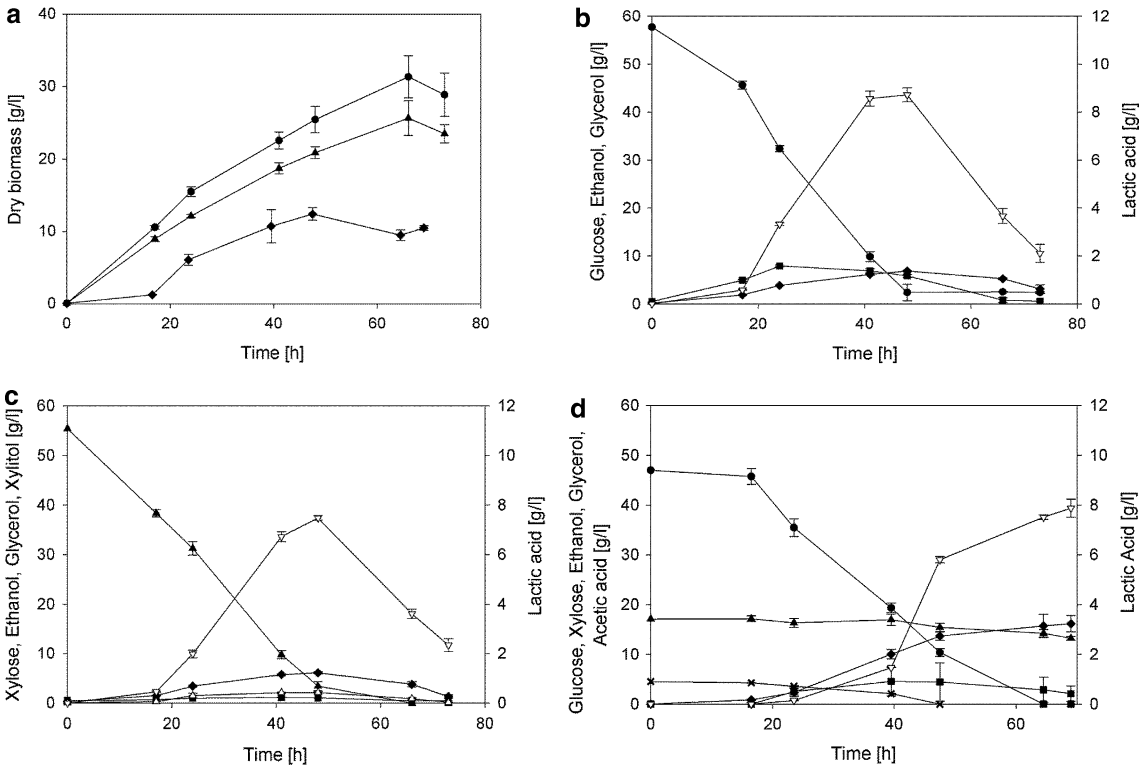

Fig. 6 **a** Growth curves of *C. lignohabitans*-LDH on YP medium supplemented with glucose 5 % (*filled circles*), xylose 5 % (*filled triangles*) and lignocellulosic hydrolysates (5 % total sugar content, *filled diamonds*). **b**, **c**, **d** Metabolite profiles during the growth of *C. lignohabitans*-LDH on YPD (**b**), YPX (**c**) and on YP supplemented with lignocellulosic hydrolysates (**d**). Glucose, *filled circles*; xylose, *filled triangles*; xylitol, *open triangles*; ethanol, *filled squares*; glycerol, *filled diamonds*; acetic acid, *crosses*; lactic acid, *open inverted triangles*. (Shake flasks, 30 °C)

itaconic acid amount, compared to other genetically engineered microorganisms [21]. These results represent therefore an encouraging starting point for the development of a new microbial cell factory for itaconic acid production.

Lactic acid production

Lactic acid, another industrially relevant chemical, was chosen as target product, to further investigate the versatility of this microorganism for conversion of lignocellulosic biomass into industrially relevant chemicals.

L-Lactate dehydrogenase (L-LDH; EC 1.1.1.27) is the enzyme responsible for conversion of pyruvate into L-lactic acid, with the concomitant reduction of NAD^+. The *Lactobacillus plantarum* LDH gene has proven to be the most efficient heterologous gene for the production of lactic acid in *S. cerevisiae* [5]. This coding sequence was therefore chosen for the expression in *C. lignohabitans*, placed under the control of the isolated endogenous pGAP and GAPtt.

Several of the obtained transformants were cultivated on glucose. All the clones showed a growth rate comparable to the wild type, showing that the expression of the lactate dehydrogenase does not significantly affect the general growth of the strain (data not shown). Lactic acid was accumulated to concentrations ranging from 4 to 10 g/l. We speculate that these different performances could be attributed to a different number of copies of the transformed construct integrated into the genome, or to the integration of the construct into differentially transcribed regions of the genome.

The trends of glucose, lactic acid, ethanol and glycerol for the best performing clone are shown in Fig. 6b. Interestingly, lactic acid was the predominant end product of the metabolism (as already reported for *Pichia stipitis* [12]). Only after depletion of glucose, the previously accumulated lactic acid, ethanol and glycerol were consumed. In this sense, glucose has probably a catabolite repression effect on the consumption of other carbon sources. However, the depletion of lactic acid confirms the presence of an endogenous pathway for the reoxidation of lactic acid in *C. lignohabitans*, which will have to be carefully considered to avoid the consumption of the desired product in a production process.

The transformants were cultivated on xylose. Also in this case, the transformants' growth rate was not significantly different from the wild type, and lactic acid reached

concentrations between 2.5 and 7.5 g/l. The lactic acid concentrations reached by each transformant on xylose correlate with the ones obtained from the same strains on glucose, confirming the reproducibility of the transformants' performances. Furthermore, during growth on xylose the predominant metabolite accumulated was lactic acid, over the natural products ethanol, glycerol and xylitol (results shown in Fig. 6c). The overall yield of lactic acid on xylose (0.145 g/g) was lower than on glucose (0.163 g/g), indicating that the metabolic flux through the fermentative pathways is weaker during the growth on xylose. This is also consistent with the observation that the ethanol yield on glucose is higher than on xylose.

To evaluate the possibility of producing lactic acid starting from lignocellulosic biomass, the best performing *C. lignohabitans* clone was cultivated on rich medium supplemented with wood chips' hydrolysates. Glucose (47 g/l) and xylose (17 g/l) were the main sugars present in the hydrolysates; mannose, galactose and arabinose were not detectable. Inhibiting compounds such as acetic acid (4.5 g/l), furfural (0.43 g/l) and HMF (0.09 g/l) were also observed in the lignocellulosic hydrolysates. As previously shown (Fig. 2), *C. lignohabitans* showed no inhibition in growth on hydrolysates of different lignocellulosic sources, compared to the growth on pure sugars. However, the biomass accumulated by the *C. lignohabitans*-LDH strain during the growth on hydrolysates was considerably less than the biomass grown on glucose or xylose (Fig. 6a). We suppose here a cumulative toxic effect given by the accumulation of lactic acid in presence of acetic acid. The accumulation of lactic acid in the medium decreases the pH of the culture, bringing the acetate into its non-dissociated form. Acetic acid is known to have a strong negative effect on cellular vitality [20, 29], and we demonstrated that the presence 5 g/l of acetic acid at pH 3 was able to inhibit the growth of *C. lignohabitans* (data not shown), as already reported for other related yeasts such as *C. shehatae* and *P. stipitis* [17]. In this context of reduced strain fitness, a complete glucose consumption occurred only after 65 h of growth and xylose was not fully consumed when the experiment was stopped (Fig. 6d). The strain was however able to accumulate lactic acid with a concentration up to 8 g/l, offering a proof of concept of the ability of engineered *C. lignohabitans* to convert sugars from lignocellulosic biomass into chemicals.

Conclusion

Efficient utilization of lignocellulosic biomass represents a challenge in biotechnology research. A microbial cell factory with all the characteristics required to fulfill this task is highly desirable. Here, we present the yeast *C. lignohabitans* as an innovative microbial host for organic acid production from lignocellulosic material. Recombinant expression of a fungal CAD or a bacterial LDH led to *C. lignohabitans* strains able to accumulate considerable amounts of itaconic and lactic acid, respectively. The data clearly show the suitability of *C. lignohabitans* as fermentative host for the production of organic acids and open the way to a further range of biorefinery applications for this yeast.

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Conflict of interest The authors declare no conflict of interest.

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