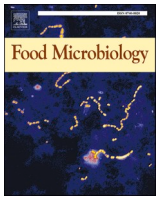
Food Microbiology 134 (2026) 104903

Contents lists available at ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm



Diversity of α-acetolactate decarboxylase in the Saccharomycotina yeast subphylum: From discovery to brewing application

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ARTICLE INFO

Keywords:
Diacetyl
Saccharomyces pastorianus
Lipomyces
Dipodascus
Wickerhamiella
Fermentation
Lagering
Beer quality
Off-flavour
Oxidative decarboxylation
α-acetolactate
Maturation period

ABSTRACT

Diacetyl, a vicinal diketone with a low sensory threshold, is a prominent off-flavour in beer, necessitating extended lagering to allow its reduction to non-flavour-active compounds. In brewing, bacterial α-acetolactate decarboxylases are commonly used to mitigate diacetyl formation by converting its precursor, α-acetolactate, directly into acetoin. Here, we report the first discovery and characterization of functional α-acetolactate decarboxylases enzymes of eukaryotic origin, specifically from yeasts within the Saccharomycotina subphylum. Using a homology-based search against fungal genomic databases, 29 candidate genes were identified across 18 yeast species from only three genera (*Lipomyces*, *Dipodascus* and *Wickerhamiella*) and classified into distinct phylogenetic groups. Phylogenetic analysis revealed both fungal and possible bacterial origins, suggesting evolutionary conservation and horizontal gene transfer events. Seven genes were heterologously expressed in *Saccharomyces pastorianus* lager brewing strains. Fermentation trials in both lab-scale septum flasks and E.B.C. tall tubes demonstrated that yeast-derived α-acetolactate decarboxylases significantly reduced diacetyl levels, with some performing comparably or superior to the benchmark *Brevibacillus brevis* enzyme. These strains also showed normal fermentation kinetics and produced beers with diacetyl concentrations below sensory thresholds, effectively eliminating the need for extended lagering. Our findings uncover a previously unrecognized enzymatic activity in budding yeasts and present yeast α-acetolactate decarboxylases as promising non-bacterial alternatives to improve process efficiency and sustainability in lager beer production.

1. Introduction

Diacetyl is a vicinal diketone that imparts a buttery flavour and aroma, essential in dairy products such as butter, buttermilk, and fresh cheeses at low concentrations (Hugenholtz and Starrenburg, 1992). However, in the context of brewing, diacetyl is considered a significant off-flavour, detrimental to the quality of the final product (Krogerus and Gibson, 2013a). This compound is produced as a by-product of fermentation by various microorganisms, including the lager brewing yeast *Saccharomyces pastorianus*. Diacetyl is produced during fermentation through the oxidative decarboxylation of α-acetolactate, an intermediate in the biosynthesis of the amino acids leucine and valine (Fig. 1). *Saccharomyces pastorianus* produces diacetyl under normal fermentation conditions (Suomalainen and Ronkainen, 1968). The diacetyl concentration can impart off-flavours even at minimal concentrations, as it is characterized by its low sensory threshold, ranging from 17 to 70 μg L^{-1} (Meilgaard, 1982; Saison et al., 2009). Similarly, the vicinal diketone 2,3-pentanedione is also produced by oxidative decarboxylation, but of (S)-2-aceto-2-hydroxybutanoate, intermediate of isoleucine pathway. However, 2,3-pentanedione sensory threshold is an order of magnitude higher (900–1000 μg L^{-1}) than that of diacetyl and therefore does not pose any sensory disturbance in brewing products (Meilgaard, 1975; Wainwright, 1973). Although diacetyl is produced during fermentation, it can be reassimilated and reduced to less flavour-active compounds such as acetoin and 2,3-butanediol by *S. pastorianus* (Masschelein, 1986). This reduction process is key as it influences the overall length of the fermentation. To achieve the low diacetyl concentrations necessary for high-quality beer, an additional maturation period, known as lagering, is required for green beer (Gorter de Vries et al., 2019). The requirement for an extended lagering period represents a significant bottleneck for breweries. This maturation stage not only occupies fermentation vessels for longer durations but also incurs additional energy costs, impacting overall production efficiency (D'Ascenzo et al., 2024). Strategies to minimize diacetyl levels more

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https://doi.org/10.1016/j.fm.2025.104903
Received 8 July 2025; Received in revised form 12 August 2025; Accepted 14 August 2025
Available online 18 August 2025

rapidly could provide substantial benefits in terms of operational efficiency and cost-effectiveness (Krogerus and Gibson, 2013a).

Since the correlation between diacetyl and beer quality is of key importance, this subject has received a lot of attention and many approaches to reduce lagering time have been devised which fall in two categories, i) those aiming at optimizing process conditions and ii) those aiming at improving the yeast. The first category encompasses strategies aiming at establishing wort recipes balanced in branched chain amino acid concentration. Enriching wort with valine leading to faster uptake resulted in most cases in lower diacetyl, since valine acted as an allosteric effector that feedback inhibits α-acetolactate synthase activity and thus preventing *de novo* biosynthesis of α-acetolactate (Magee and Derobich, 1968a,b; Barton and Slaughter, 1992; Krogerus and Gibson, 2013b). Combining increased valine concentration with a reduction of free amino nitrogen also led to lower diacetyl, likely caused by the stimulation of the valine uptake rate (Nakatani et al., 1984; Petersen et al., 2004). Modifications of the mashing and kilning steps in wort preparation have also been linked to variation in final diacetyl (Jones, 2005; Schwarz et al., 2012). Increasing temperature towards the end of the fermentation can be exploited to fully convert diacetyl to acetoin enabling the shortening of the maturation phase (Saerens et al., 2008). While in most cases diacetyl was lower or reduced faster, its formation was not eliminated. The second category comprises strategies aiming at controlling the diacetyl formation by improving metabolic function of the fermenting yeast e.g. *S. pastorianus*. The main strategies targeted the α-acetolactate node by either preventing its formation or by accelerating its utilization. The diminution of the copy number of *ILV2*, gene that encodes for the catalytic subunit of the α -acetolactate synthase in *S. pastorianus,* was associated with a reduction of diacetyl level (Zhang et al., 2008; Shi et al. 2016, 2017). Similarly, reduction of the copy number of *ScILV6* that encodes the regulatory subunit of the α -acetolactate synthase resulted in a significant decrease of diacetyl (Duong et al., 2011). Complementary approaches that targeted the reactions downstream α-acetolactate which included overexpression of *ILV5,* gene encoding the acetohydroxyacid reductoisomerase alone or in combination with *ILV3* which encodes the dihydroxyacid dehydratase, demonstrated reduction of the maximum diacetyl produced but were not sufficient to completely eliminate lagering (Dillemans et al., 1987; Gjermansen et al., 1988; Kusunoki and Ogata, 2012). However, out of the envisaged strategies, the most promising consisted in expressing a heterologous α-acetolactate decarboxylase, an enzyme that catalyses the non-oxidative decarboxylation of α-acetolactate into acetoin (Loken and Stormer, 1970; Diderichsen et al., 1990). Overexpression of an α-acetolactate decarboxylase from *Enterobacter aerogenes*, *Klebsiella terrigena* (Blomqvist et al., 1991) or *Acetobacter aceti* (Yamano et al., 1994) in brewer's yeast led to a massive reduction of the extracellular diacetyl concentration to the extent that the lagering phase could be eliminated (Fig. 1). To circumvent the inconvenience that such a strain is genetically modified and might limit public acceptance, the enzyme can be directly added to wort to enhance the conversion of residual extracellular α-acetolactate in acetoin. Typically, for this application, enzymes from *Bacillus* species such as *Brevibacillus brevis* and *Bacillus licheformis* are used (Diderichsen et al., 1990; Silano et al., 2018).

The α-acetolactate decarboxylase described in scientific literature and applied in brewing are from bacterial origin exclusively. With the advancement in sequencing technology in an attempt to broaden the diversity of enzyme, this study explored whether such enzyme could be found in eukaryotes and more specifically in yeasts of the Saccharomycotina subphylum. For this, interrogation of both protein and nucleotide databases was performed. New yeast sequences were then compared to available biodiversity of the α acetolactate enzyme. This enabled to assume the evolutionary origin of these novel enzymes. A

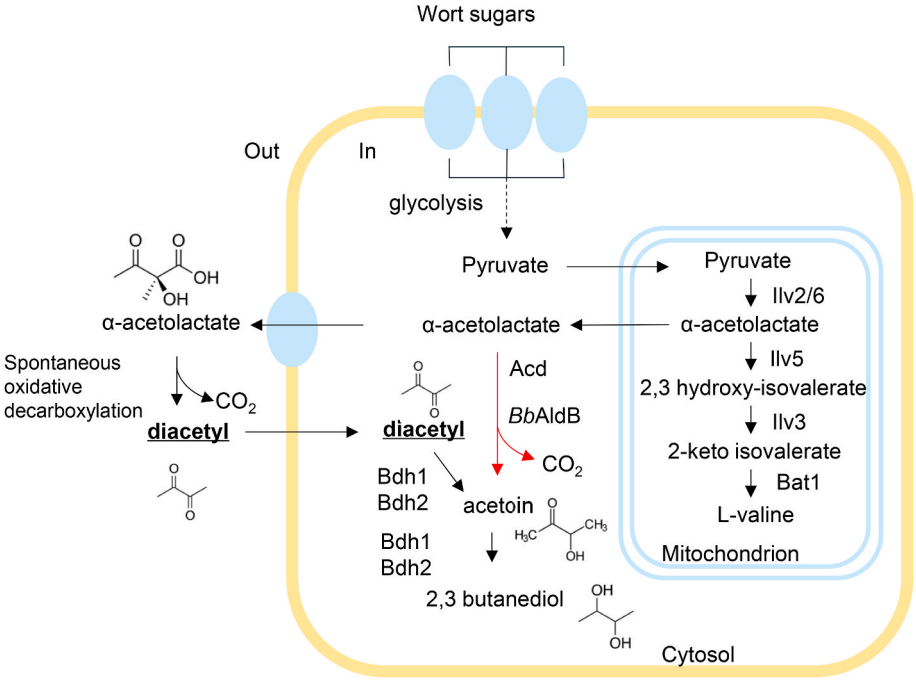

Fig. 1. Pathway for diacetyl formation and reduction in *Saccharomyces* species and the role of α-acetolactate decarboxylase as a diacetyl bypass.
A portion of the pyruvate generated from glycolysis of wort sugars is imported into the mitochondria and directed into the branched-chain amino acid (BCAA) biosynthetic pathway. Here, the valine biosynthesis branch is shown as an example. In the first step, α-acetolactate is formed by Ilv2/Ilv6 (α-acetolactate synthase). This intermediate is either further processed by Ilv5 within the BCAA biosynthesis pathway or, to a lesser extent, escapes into the extracellular environment, where it undergoes spontaneous decarboxylation to form diacetyl. Once reabsorbed by the cell, diacetyl is sequentially reduced to acetoin and then to 2,3-butanediol by the dehydrogenases Bdh1 and Bdh2. The α-acetolactate decarboxylase (Acd) bypass (shown in red) converts transient cytosolic α-acetolactate directly into acetoin, thereby preventing diacetyl accumulation. Ilv2/Ilv6 (Acetolactate synthase/Regulatory subunit of acetolactate synthase), Ilv5 (Acetohydroxyacid reductoisomerase), Ilv3 (Dihydroxyacid dehydratase), Bat1 (Mitochondrial BCAA aminotransferase), Acd (α-acetolactate decarboxylase), *Bb*Ald (*Brevibacilus brevis* α-acetolactate decarboxylase (Svendsen et al. 1989)). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

selected set of eukaryotic putative α-acetolactate decarboxylases was functionally characterised by overexpression in *S. pastorianus* to monitor their ability to control diacetyl formation during wort fermentation.

2. Materials and methods

2.1. Strains and growth media

All *S. pastorianus* strains used in this study are listed in Table 1 *S. pastorianus* strains CBS 1513 and CBS 1483 were obtained from the Westerdijk Fungal Biodiversity Institute (https://wi.knaw.nl/). *Escherichia coli* XL1 blue (Agilent Technologies, Santa Clara, CA) was used for plasmid propagation. *S. pastorianus* and *E. coli* strains were stored at −80 °C in 30 % glycerol (v/v).

S. pastorianus strains were cultivated in complex YPD medium (10 g L^{-1}yeast extract, 20 g L^{-1}peptone, 20 g L^{-1} glucose). When required, YPD was supplemented with 200 mg L^{-1} hygromycin B. *S. pastorianus* strains were grown at 20 °C, 200 rpm in an Innova 43/43R incubation shaker (Brunswick, Nijmegen, the Netherlands) or at 20 °C in a temperature controlled room on solid media. Fermentations were performed at 12 °C with whole malt wort at either 17 °P or 5.7 °P (Heineken, Zoeterwoude, The Netherlands) (Bennis et al., 2023). Whole malt wort of 5.7 °P was supplemented with 1 mL L^{-1} pluronic acid. *E. coli* strains were grown in lysogeny broth (LB) supplemented with 100 mg L^{-1} ampicillin (LB-amp), 50 mg L^{-1} kanamycin (LB-kan) or 10 mg L^{-1} chloramphenicol (LB-cam) at 37 °C, 200 rpm in an Innova 4000 Incubator Shaker (Eppendorf AG, Hamburg, Germany). Solid media was prepared by adding 20 g L^{-1} Bacto Agar to liquid media.

2.2. Molecular biology techniques

DNA templates for cloning were amplified with Phusion high-fidelity polymerase (Thermo Fisher Scientific, Landsmeer, Netherlands) according to manufacturer's protocol. Diagnostic PCR was performed with DreamTaq PCR mastermix (Thermo Fisher Scientific). Plasmids and primers used in this study are shown in Tables S1 and S2. Constructed plasmids were transformed to chemical competent *E. coli* XL1 blue and grown under selective conditions. PCR results were analysed by electrophoresis on a 1 % agarose gel. GenElute plasmid miniprep kit (Sigma-Aldrich, Saint Louis, MO) was used to isolate the plasmids from *E. coli*.

Table 1
Saccharomyces pastorianus used in this study.

Strain	Genotype	
CBS 1513	Group I *S. carslbergensis*-type strain; Carsberg brewery; bottom yeast no. I; isolated in 1883	Dunn and Sherlock (2008)
CBS 1483	Group II bottom yeast; Brewery-Heineken; isolated July 1927	Salazar et al. (2019)
IMI483	CBS1483 Δ*Sc*YCR087C::*ScTDH3*p-*BbaldB-ScENO1*t	Bennis et al. (2023)
IMI603	CBS1483 Δ*Se*YCL036W::*ScTDH3*p-*WvACD2*$_{JCM5958}$-*ScENO2*t	This study
IMI604	CBS1483 Δ*Se*YCL036W::*ScTDH3*p-*LaACD1*-*ScENO2t*	This study
IMI609	CBS1513 Δ*Sc*YCR087C::*ScTDH3*p-*BbaldB-ScENO1t*	This study
IMI610	CBS1513 Δ*Sc*YCR087C::*ScTDH3*p-*WvACD2*$_{JCM5958}$-*ScENO2t*	This study
IMI611	CBS1513 Δ*Sc*YCR087C::*ScTDH3*p-*LaACD1*-*ScENO2t*	This study
IMI630	CBS1513 Δ*Sc*YCR087C::*ScTDH3*p-*WaACD1*-*ScENO2t*	This study
IMI631	CBS1513 Δ*Sc*YCR087C::*ScTDH3*p-*WdACD1*$_{NRRLY-6692}$-*ScENO2t*	This study
IMI632	CBS1513 Δ*Sc*YCR087C::*ScTDH3*p-*WvACD1*$_{JCM5958}$-*ScENO2t*	This study
IMI633	CBS1513 Δ*Sc*YCR087C::*ScTDH3*p-*DfACD1*-*ScENO2t*	This study

2.3. Construction of plasmids and CRISPR-Cas9 repair fragments

The expression cassettes used in this study were constructed using the Yeast Toolkit (Lee et al., 2015). Genes encoding Acd1 were codon optimized for *S. cerevisiae* using the Jcat Codon Adaptation tool (Grote et al., 2005). Type 3 part specific flanking regions containing *Bsm*BI and *Bsa*I restriction sites were added to the sequence and the genes including the flanking regions were ordered as synthetic genes (GeneArt, Thermo Fisher Scientific), resulting in part plasmids pGGKp414 (*WvACD2*$_{JCM5958}$), pGGKp415 (*LaACD1*), and pGGKp438-pGGKp441 (*WaACD1*, *WdACD1*$_{NRRLY-6692}$, *WvACD1*$_{JCM5958}$, *DfACD1* respectively). Using Golden Gate assembly with the restriction enzyme *Bsa*I the left connector (pYTK002, ConLS), promoter (p*TDH3*, pYTK009), *ACD1* gene (part plasmids type 3), terminator (t*ENO2*, pYTK055), and right connector (pYTK072, ConRE) fragments were assembled in the pYTK095 entry vector resulting in expression cassettes containing one *ACD1* transcriptional unit (pUD1359 (*WvACD2*$_{JCM5958}$), pUD1360 (*LaACD1*), and pUD1419-pUD1422 (*WaACD1*, *WdACD1*$_{NRRLY-6692}$, *WvACD1*$_{JCM5958}$ and *DfACD1*, respectively)).

GFP dropout plasmids were constructed to contain homology arms corresponding to Sc-SeCHRIII landing sites found in *S. pastorianus* strains (Bennis et al., 2023). For *Se*YCL036W homology, the left homology arm was PCR amplified from the CBS 1483 genome using primers 19952 and 19953 with YTK part 8b specific overhangs. The right homology arms were amplified from CBS 1483 genome using primers 19954 and 19955 with YTK part 67 specific overhangs. Both homology arms were integrated in the pYTK001 entry vector using Golden Gate cloning with restriction enzyme *Bsm*BI, resulting in pGGKp383 and pGGKp384. Golden Gate assembly of pYTK008, pYTK047, pYTK073, pYTK090, pGGKp383, pGGKp384 with restriction enzyme *Bsa*I resulted in GFP dropout plasmids pGGKd089. The same method was applied for the construction of the GFP dropout plasmid containing *Sc*YCR087C homology arms. Primers 18871 and 18872 were used for the construction of the YTK type 8b left homology arm, and primers 18873 and 18874 for the construction of the YTK type 67 right homology arm. Both homology arms were integrated in the pYTK001 entry vector using Golden Gate cloning with restriction enzyme *Bsm*BI, resulting in pGGKp343 and pGGKp344. Golden Gate assembly of pYTK008, pYTK047, pYTK073, pYTK090, pGGKp343, pGGKp344 with restriction enzyme *Bsa*I resulted in GFP dropout plasmids pGGKd092. The GFP dropout plasmids were used to construct integration plasmids using Golden Gate cloning with the restriction enzyme *Bsa*I. This resulted integration plasmids carrying the repair fragments for the integration of *WvACD2*$_{JCM5958}$ in *Se*YCL036W (pUDI309) or *Sc*YCR087C (pUDI346), and *LaACD1* in *Se*YCL036W (pUDI310). All plasmids were verified by diagnostic PCR using the primers specified in Table S2 followed by sequencing. Plasmid Sequencing was performed by Plasmidsaurus (Monrovia, CA) using Oxford Nanopore Technology (Oxford, United Kingdom).

2.4. Construction of yeast strains

CRISPR-Cas9 genome editing in *S. pastorianus* strains was performed as described in (Gorter de Vries et al., 2017). Plasmids carrying the repair fragments for the integration of *WvACD2*$_{JCM5958}$ in *Se*YCL036W (pUDI309) or *Sc*YCR087C (pUDI346), and *LaACD1* in *Se*YCL036W (pUDI310) were digested with *Not*I to release the repair fragments. For the construction of the repair fragments for integration of the remaining *ACD1* genes in *Sc*YCR087C, the repair fragments were PCR amplified with primers containing integration site specific homology arms. The *BbaldB* expression cassette was amplified from pUD1218 with primer pair 18532 and 18533. The *LaACD1*, *WaACD1*, *WdACD1*$_{NRRLY-6692}$, *WvACD1*$_{JCM5958}$, and *DfACD1* expression cassettes were amplified with primer pair 18532 and 20679 from pUD1360, pUD1419, pUD1420, pUD1421, and pUD1422 respectively. The DNA fragments were purified using a GeneJET PCR Purification kit (Thermo Fisher Scientific). 1000

ng repair fragment was co-transformed with 500 ng *Spy*Cas9-gRNA plasmid by electroporation as described previously (Bennis et al., 2023). The transformed cells were recovered in YPD for 2h at 20 °C in a temperature controlled room and plated on selective medium. Gene integrations in *Sc*YCR087C (for expression in CBS 1513) or *Se*YCL036W (for expression in CBS 1483) were confirmed by PCR (Bennis et al., 2023). For these PCR, the lithium acetate (LiOAc)-SDS method was used to isolate yeast genomic DNA template (Looke et al., 2011). The transformants were further verified by whole genome sequencing.

2.5. Whole genome sequencing

Yeast genomic DNA of the transformants was isolated using QIAGEN Genomic-tip 100/G kit (Qiagen, Hilden, Germany). Genomic DNA concentrations were measured with the BR ds DNA kit (Invitrogen, Carlsbad, CA) using a Qubit 2.0 Fluorometer (Thermo Fisher Scientific). Library preparation and whole genome sequencing was done by Macrogen Europe (Macrogen Europe BV, Amsterdam, The Netherlands). DNA libraries were prepared using the TruSeq DNA PCR-Free Library Preparation Kit, and sequenced using NovaseqTM (Illumina; San Diego, CA). Sequencing data are available under bioproject PRJNA1282013 at NCBI (https://www.ncbi.nlm.nih.gov/) (Table S3). The sequencing reads were mapped to the CBS 1483 or CBS 1513 genome (Salazar et al., 2019; Walther et al., 2014) including expected engineered contigs using the Burrows-Wheeler (BWA) tool (Li and Durbin, 2010) and further processed using SAMtools (Danecek et al., 2021; Li et al., 2009). Chromosomal copy number was estimated by the Magnolya algorithm (Nijkamp et al., 2012) (Table S4). The sequences were analysed by visualizing the bam files in the Integrative Genomics Viewer (IGV) software (version 2.8.9) (Thorvaldsdóttir et al., 2013).

2.6. Fermentation in septum flasks

Frozen aliquots of CBS 1513, IMI609-IMI611 and IMI630-IMI633 were inoculated in 100 mL YPD medium in 500 mL shake flasks and grown at 12 or 20 °C, 200 rpm for 3–5 days. These cultures were used to inoculate 500 mL shake flasks with 100 mL YPD and grown at 12 °C, 200 rpm until exponential growth. The exponential growing cells were washed in sterile dH_2O and used to inoculate 60 mL whole malt wort (5.7 °P) at a starting dilution of 0.5 mL^{-1} in 100 mL bottles sealed with rubber stopper septum (Diderich et al., 2018). The cultures were incubated at 12 °C, 200 rpm for nine days with daily sampling to determine cell density (OD_{660nm}), sugar consumption, ethanol production, and vicinal diketone (diacetyl, 2,3-pentadione) concentrations (Brickwedde et al., 2018).

2.7. Fermentation in E. B. C. Tall tubes

Frozen aliquots of CBS 1513, IMI609-IMI611, and IMI631 were inoculated in 100 mL YPD medium in 500 mL shake flasks and grown at 12 or 20 °C, 200 rpm for 3–5 days. These cultures were used to inoculate four times 500 mL shake flasks with 100 mL YPM (maltose, 6 %) and grown at 12 °C, 200 rpm until exponential growth. Exponentially growing cells were washed in sterile dH_2O and transferred into 2.25 L (singles) or 5 L (duplicates) whole malt wort (17 °P) to obtain a starting cell density of approximately $5 \times 10^{+6}$ cells mL^{-1}. E. B. C. tall tubes were filled with 2.25 L of the inoculum and equipped with Bronkhorst EL-FLOW® Prestige mass flow meters (Bronkhorst, Veenendaal, The Netherlands) for online CO_2 measurements (Bennis et al., 2024). The fermentations were performed at 12 °C using cryostats for cooling. Samples for cell counts, °Plato, pH, sugar, ethanol, esters, and vicinal diketone concentration determinations were taken regularly.

2.8. Analytical measurements

Maltotriose, maltose, glucose, fructose, glycerol and ethanol were measured using HPLC (Agilent Technologies, Santa Clara, CA, 1260 HPLC system) equipped with an Bio-Rad HPX-42A column (300 × 7.8 mm, 25 μm) (Bio-Rad, Hercules CA) operating at 75 °C using MilliQ as eluent at a flow rate of 0.5 mL·min−1 for 30 min. Compounds were measured using an 1260 Refractive Index Detector (RID) at 35 °C. Samples were 0.2 μm filter-sterilized before analysis (Brickwedde et al., 2017).

Diacetyl and 2,3-pentanedione levels were analysed using static headspace gas chromatography with a 7890A Agilent GC with an electron capture detector on an Agilent CP-Sil 8 CB (50 m × 530 μm x 1 μm) capillary column. A split flow of 8 mL N_2 min^{-1} with a split ratio of 1:1 was used. The injector temperature was set at 120 °C and an oven temperature profile of 35 °C followed by an increase of 30 °C min-1 to 150 °C was used. The ECD temperature was set at 150 °C with a make-up flow of 30 mL N_2 min^{-1}.

Ester and higher alcohols were analysed using static headspace gas chromatography with a flame ionization detector FID (Agilent technologies 7890A) and a DB-WAXetr capillary column (30 m × 320 μm x 1 μm). 2.5 mL of culture supernatant was heated to 50 °C for 5 min prior to injection using a CTC Analytics Combi Pal headspace auto-injector. A split flow of 9.88 mL N_2 min^{-1} with a split ratio of 5:8:1 The injector temperature was set at 250 °C and an oven temperature profile of 55 °C at the start followed by an increase of 20 °C min^{-1} to 160 °C with a hold time of 4.75 min^{-1} was used. The FID temperature was set at 250 °C with a make-up flow of 10 mL hydrogen min^{-1}.

2.9. Identification and homology assessment of putative Acd1 proteins in budding yeasts

The amino acid sequence of *Brevibacillus brevis* AldB (WP_064202791; Uniprot ID: P23616-ALDC_BREBE) (Diderichsen et al., 1990) was used to perform a tblastn similarity searches against the translated whole-genome sequencing data of the Saccharomycotina subphylum as database (Altschul et al., 1997). The translation of the identified nucleotide sequences were aligned using ClustalW (Thompson et al., 1994). The resulting identity matrix was used to draw a heat map displaying sequence similarity with GraphPad Prism (Boston, MA).

2.10. Phylogenetic tree

To build the α-acetolactate decarboxylase phylogenetic tree, a search using as query the EC number of α-acetolactate decarboxylase (E.C. 4.1.1.5) retrieved a total of 5647 protein sequences. A first filter was applied to reduce redundancy at species level. If for a single species several sequences showed length differences, a sequence corresponding to each group was conserved. Secondly, the 29 novel Saccharomycotina sequences were added to the sequence set. In total 2517 characterized and putative α-acetolactate decarboxylase amino acid sequences (Table S5a and S5b) were aligned using Muscle (Edgar, 2004). The phylogenetic file output was used in iTOL3 for representation and annotation (Letunic and Bork, 2024).

3. Results

Uncovering putative α-acetolactate decarboxylase in Saccharomycotina yeasts.

To date, the functional characterization of α-acetolactate decarboxylase has primarily focused on enzyme of prokaryotic origins. In a search to investigate the presence of such enzymatic activity within yeasts of the Saccharomycotina subphylum, we employed the *Bb*AldB *Brevibacillus brevis* protein sequence (WP_064202791) (Diderichsen et al., 1990) as a query to survey translated whole-genome sequencing data of budding yeasts with tblastn (Johnson et al., 2008). The analysis identified 37 assembled contigs potentially harbouring an α-acetolactate decarboxylase gene. Upon initial inspection, five contigs were flagged for sequence sample contamination, while three contigs appeared to be

duplicated within the NCBI database. However, the remaining 29 contigs (Table 2) were confirmed to originate from the intended sequenced yeast species. The translation of the putative α-acetolactate decarboxylase genes included in the found contigs, exhibited a sequence identity with the *Bb*AldB sequence ranging from 31 to 36 %. Markedly, these sequences stemmed from 18 yeast species belonging to the Lipomycetaceae (*Lipomyces*), Trichomonascaceae (*Wickerhamiella*), and Dipodascaceae (*Dipodascus*) families. While this analysis hinted at the potential occurrence of α-acetolactate decarboxylase in budding yeasts, it appeared limited to a specific range of species.

Alignment of the 29 amino acid sequences showed that the putative α-acetolactate decarboxylase protein from *Wickerhamiella azyma* (14a_WaPYCC 8333_20) exhibited less than 30 % identity relative to the other yeast sequences forming a group on its own (Group 1) (Fig. 2, and Table 2). Based on the multi-sequence alignment, the rest of the sequences were segregated in three additional groups; Group 2 included proteins from three *W. versatilis* strains, Group 3 the largest of all, included proteins from *Lipomyces* species and *Dipodascus fermentans* and

Table 2
TBlastn results of the search of the whole genome sequence shotgun database at NCBI limited to data from organisms of the Sacchaormycotina subphyllum using the *Brevibacillus brevis* α -acetolactate decarboxylase *Bb*AldB as query. The retrieved contigs as well as the strain accession number of the genomic data and AA sequence identity to *Bb*AldB are provided. Group mentions refer to Fig. 2.

Contig	Organism	Strain	Bioproject	Biosample	Systematic Protein name	Proposed gene name	Group		Identity with the query *Bb*AldB (%)
JAKISA010000020.1	*Wickerhamiella azyma*	PYCC 8333	PRJNA794368	SAMN24648683	14a_WaPYCC 8333_20	*WaACD1*	1	Prokaryote	31.250
JAQOWU010000008.1	*Wickerhamiella versatilis*	HR3	PRJNA929327	SAMN32957171	17a_WvHR3_2	*WvACD2*	2	Prokaryote	35.945
NRED01000011.1	*Wickerhamiella versatilis*	NRRL Y-6652	PRJNA396763	SAMN07511540	17d_WvNRRL Y-6652_11	*WvACD2*	2	Prokaryote	35.945
BCJV01000004.1	*Wickerhamiella versatilis*	JCM 5958	PRJDB3712	SAMD00028433	17e_WvJCM 5958_3	*WvACD2*	2	Prokaryote	35.945
LFTX01003299.1	*Dipodascus fermentans*	CICC 1368	PRJNA287202	SAMN03777305	1a_DfCICC 1368_8094	*DfACD1-1*	3	Eukaryote	31.628
LFTX01001780.1	*Dipodascus fermentans*	CICC 1368	PRJNA287202	SAMN03777305	1b_DfCICC 1368_3782	*DfACD1-2*	3	Eukaryote	32.432
PPJT02000222.1	*Lipomyces arxii*	NRRL Y-17921	PRJNA429441	SAMN08343392	2a_LaNRRL Y-17921_222	*LaACD1*	3	Eukaryote	32.766
JAKVQM010000031.1	*Lipomyces chichibuensis*	CBS 12929	PRJNA736342	SAMN20341218	3a_Lc_CBS 12929_31	*LcACD1*	3	Eukaryote	32.877
JAJLTN010000208.1	*Lipomyces kockii*	NRRL Y-27505	PRJNA736342	SAMN20341722	4a_Lkoc_NRRL Y-27505_535	*LkocACD1*	3	Eukaryote	32.110
JAJMAF010000119.1	*Lipomyces kononenkoae*	NRRL Y-63818 T	PRJNA736342	SAMN20341916	5a_LkonNRRL Y-63818_119	*LkonACD1*	3	Eukaryote	33.790
PPJS03000083.1	*Lipomyces mesembrius*	NRRL Y-27506	PRJNA429441	SAMN08343393	6a_LmNNRL Y-27506_84	*LmACD1*	3	Eukaryote	32.906
JAJLTP010000052.1	*Lipomyces orientalis*	NRRL Y-27927	PRJNA736342	SAMN20341935	7a_LoNRRL Y-27927_102	*LoACD1*	3	Eukaryote	35.616
PPJW02000004.1	*Lipomyces* sp.	NRRL Y-11553	PRJNA429441	SAMN08343389	8a_LspNRRL &_11553_4	*LspACD1*	3	Eukaryote	31.197
JAJLTQ010000062.1	*Lipomyces spencermartinsiae*	NRRL Y-7042	PRJNA736342	SAMN20341935	9a_LspenNRRL Y7042_121	*LspeACD1*	3	Eukaryote	36.530
JAJMAE010000075.1	*Lipomyces spencermartinsiae*	NBRC 10376	PRJNA736342	SAMN20341399	9b_LspenNBRC 10376_75	*LspeACD1*	3	Eukaryote	32.489
JAKTPT010000205.1	*Lipomyces starkeyi*	NRRL Y-11557	PRJNA736342	SAMN20341431	10a_LstaNRRL Y-11557_207	*LsACD1*	3	Eukaryote	33.028
LSGR01000298.1	*Lipomyces starkeyi*	NRRL Y-11557	PRJNA71653	SAMN00794676	10b_LstaNRRL Y-11557_298	*LsACD1*	3	Eukaryote	33.028
JAGJTG010000012.1	*Lipomyces starkeyi*	AS 2.1560	PRJNA719292	SAMN18594842	10c_LstaAS 21560_12	*LsACD1*	3	Eukaryote	33.028
JAJLTR010000018.1	*Lipomyces tetrasporus*	NRRL Y-11562	PRJNA736342	SAMN20341935	11a_LtNRRL Y-11562_33	*LtACD1*	3	Eukaryote	36.530
JARPMG010000007.1	*Lipomyces tetrasporus*	NRRL Y-64009	PRJNA928472	SAMN32935022	11b_LtNRRL Y-64009_7	*LtACD1*	3	Eukaryote	36.530
JAKTPU010000006.1	*Lipomyces yamadae*	NRRL Y-27507	PRJNA736342	SAMN20341723	12a_LyamNRRL Y-27507_6	*LyamACD1*	3	Eukaryote	33.028
JANJPG010000597.1	*Lipomyces yarrowii*	CBS 7557	PRJNA736342	SAMN20341228	13a_LyarCBS 7557_598	*LyarACD1*	3	Eukaryote	31.064
JAKTWK010000002.1	*Wickerhamiella bombiphila*	NRRL Y-27640	PRJNA736342	SAMN20341755	15a_WbNRRL Y-27640_2	*WbACD1*	4	Eukaryote	31.308
PEOA01000124.1	*Wickerhamiella domercqiae*	PYCC 3067	PRJNA416493	SAMN07958197	16a_WdPYCC 3067_299	*WdACD1*	4	Eukaryote	33.778
NREC01000007.1	*Wickerhamiella domercqiae*	NRRL Y-6692	PRJNA396763	SAMN07511541	16b_WdNRRL Y-6692_7	*WdACD1*	4	Eukaryote	33.778
BCGM01000003.1	*Wickerhamiella domercqiae*	JCM 9478	PRJDB3620	SAMD00028340	16c_WdJMC 9478_2	*WdACD1*	4	Eukaryote	33.778
JAQOWU010000001.1	*Wickerhamiella versatilis*	HR3	PRJNA929327	SAMN32957171	17b_WvHR3_c1	*WvACD1*	4	Eukaryote	32.035
JAKUAR010000005.1	*Wickerhamiella versatilis*	NRRL Y-6652	PRJNA736342	SAMN20341921	17c_WvNRRL Y-6652_5	*WvACD1*	4	Eukaryote	31.602
BCJV01000003.1	*Wickerhamiella versatilis*	JCM 5958	PRJDB3712	SAMD00028433	17f_WvJCM 5958_2	*WvACD1*	4	Eukaryote	32.035

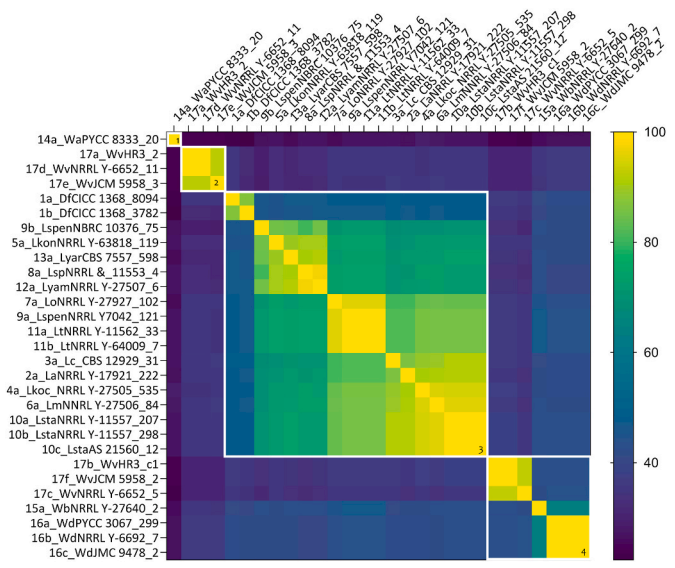

Fig. 2. Acd amino acid sequences identity. Heat map from Saccharomycotina Acd amino acid sequences identity matrix obtained by translation of open reading frame identify with tBlastn using the *Brevibacillus brevis* AldB sequence (Uniprot ID: P23616). The yeasts proteins are described in Table 2. The identity matrix was generated by multisequence alignment using Muscle (https://www.ebi.ac.uk/jdispatcher/msa/muscle?stype=protein). Denoted with white lines the yeast amino acid sequences are segregated in four groups based on sequence similarity. The group is noted in the right bottom corner of delimited square. Colours correspond to the % of identity with yellow denoting high identity and dark blue low identity. The identity scale is ranged from 100 % to 30 %. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Group 4 was composed of proteins from three *Wickerhamiella* species including *W. bombiphila*, W. *domercqiae* and *W. versatilis* (Fig. 2). While the large majority of individual genomes harboured a single α-acetolactate gene. The genome of the three *Wickerhamiella versatilis* strains (NRRL Y-6652, HR3 and JMC5958) and of *Dipodascus fermentans* that were identified, harboured two distinct putative α-acetolactate decarboxylases. While the *D. fermentans* paralogs exhibited 86.7 % identity, the *W. versatilis* paralogs shared less than 37 % identity to one another and were clustered in Group 2 for the first and in Group 4 for the second (Fig. 2 and Table 2).

3.1. Determining the origin of the yeast α -acetolactate decarboxylases

In the complex picture of yeast evolution, the discovery of a narrow distribution of the α-acetolactate decarboxylase enzyme hints at intriguing evolutionary tales. This enzyme's presence in select yeast species prompts two compelling hypotheses. One suggests an early loss of function, dating back over 300 million years ago, as Lipomycetaceae (*Lipomyces*), Trichomonascaceae (*Wickerhamiella*), and Dipodascaceae (*Dipodascus*) diverged from their yeast counterparts, as proposed (Shen et al., 2018). Alternatively, the enzyme's presence might be attributed to horizontal gene transfer events among a few yeast species. To discern between these evolutionary paths, we initially assembled a set of 2517 proteins out of an exhaustive set of 5890 entries annotated as α-acetolactate decarboxylases (EC. 4.1.1.5, https://www.brenda-enzymes.org/index.php) in Uniprot (https://www.uniprot.org/) of which 72 were from Archaeal origin, 2033 from bacterial origin and 412 from eukaryotic origin (Table S5a). This dataset covered an array of 144 taxonomic orders, illustrating the evolutionary breadth encapsulated within the enzyme's distribution. In a second step, the 29 yeast α-acetolactate decarboxylase amino acid sequences were compared with the 2517 sequences dataset. The combined 2546 sequences were aligned with Muscle (Edgar, 2004) and the alignment matrix was used to build a phylogenetic tree of the α-acetolactate decarboxylase sequences (Fig. 3 and Fig. S1).

The Archaeal sequences were distributed within the bacterial clade, which contrasted with the fungal sequences that formed a compact clade and that included 25 of the novel Saccharomycotina α-acetolactate decarboxylases, indicating that *Lipomyces*, *Dipodascus* and most of *Wickerhamiella* α-acetolactate decarboxylases originated from an eukaryotic ancestor. Out of the 25 α-acetolactate decarboxylases sequences 18 clustered together and exhibited around 44 % identity with the sequence of a putative α-acetolactate decarboxylase from *Cutaneotrichosporon oleaginosum* (A0A0J0XP78_9TREE), a basidiomycete fungus of the Trichosporonales order (Duman-Özdamar et al., 2022) and of 39 % identity with a putative α-acetolactate decarboxylase from *Ophiostoma piceae* (S3C773_OPHP1), a wood-staining fungus that grows in sapwood of conifer logs (Harrington et al., 2001). The seven remaining Saccharomycotina α-acetolactate decarboxylase sequences exhibited 50 % identity to fungal sequences derived from fungi from the Onygenales order, *Polytolypa hystricis* (A0A2B7XVP9_9EURO) (Muñoz et al., 2018) and *Ascosphaera apis* (A0A166NQ32_9EURO) (Qin et al., 2006).

However, four Saccharomycotina along four other eukaryotic α-acetolactate decarboxylase sequences seemed to have a different origin as they were placed outside the eukaryotic clade (Fig. 3) and were scattered among the bacterial sequences. The *W. azyma* α-acetolactate decarboxylase (*Wa*Acd1) was placed next to the α-acetolactate decarboxylase from a bacterium belonging to the Enterobacterales order, *Rosenbergiella nectarea* with which it shared 72 % identity. The second Saccharomycotina group segregating with prokaryotic sequences included the three *W. versatilis* sequences forming Group 2. These sequences showed a 50 % identity with an α-acetolactate decarboxylase sequence from *Zymobacter palmae* that belongs to the Oceanospirillale order (Okamoto et al., 1993).

Our analysis also identified four putative α-acetolactate decarboxylases from eukaryote origin clustering with prokaryotic enzymes. The sequence A0AA7G2CKA5_9TRY derived from the flagellated trypanosomatid protozoan *Angomonas deanei* (Teixeira et al., 2011) showed 50 % similarity with the Enterobacterales *Xenorhabdus stockiae* enzyme (A0A2D0KKI3_9GAMM). Next, two sequences from the green algae *Chlorella variabilis* (E1ZEY1_CHLVA) and *Chlorella vulgaris* (A0A9D4YTT9_CHLVU) clustering with the bacterial clade, exhibited over 40 % identity with the α-acetolactate decarboxylases sequence from *Polynucleobacter antarcticus* (A0A6M9PP41_9BURK), a planktonic freshwater bacteria. The last eukaryotic sequence (A0A0A2VZZ5_BEABA) originated from the Hypocreales fungus *Beauveria bassiana*, fungus that acts as a parasite on various arthropod species, also known as the white muscardine disease fungus. The *Beauveria* enzyme exhibited very high identity score (over 94 % identity) with the α-acetolactate decarboxylase from *Cedecea neteri* (A0A089PT51_9ENTR) and *Enterobacteriaceae bacterium* RIT693 (A0A844CIE1_9ENTR) two specimen from the Enterobacterales order.

Altogether these data demonstrated that the large majority of the yeast α -acetolactate decarboxylases had a fungal origin inherited from their last common ancestor, but in some rare circumstances the function might have been acquired through horizontal gene transfer. A mode of acquisition not only limited to Saccharomycotina yeasts but also rarely found in protozoan, filamentous fungi and algae.

To date, prokaryotic α-acetolactate decarboxylases have been inconsistently named in the scientific literature, with designations such as AldB, AldC, BudA, and AlsD. These names can lead to confusion, particularly with eukaryotic genes, where *ALD* typically refers to acetaldehyde dehydrogenases and *BUD* is associated with genes involved in budding. To avoid such ambiguities, we propose adopting the three-letter code ACD for α-acetolactate decarboxylases. This code is not currently used for any gene in *Saccharomyces cerevisiae* and was not found in gene annotations of other budding yeasts. Accordingly, Saccharomycotina genes encoding α-acetolactate decarboxylases will be designated as *ACD* (for genes) and Acd (for proteins), prefixed by the

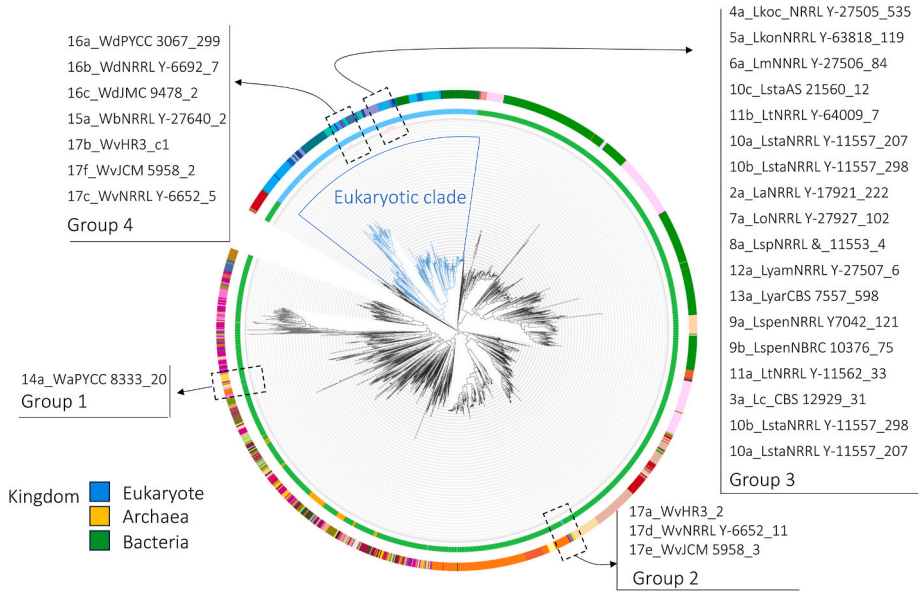

Fig. 3. Unrooted maximum likelihood phylogenetic tree of α-acetolactate decarboxylases. Proteins sequences were derived for Uniprot entries annotated with E.C. number 4.1.1.5 (https://www.brenda-enzymes.org/index). Out of the 5890 entries annotated as α-acetolactate decarboxylases, a reduction to 2517 was performed. This dataset contains 72 proteins Archaeal origin, 2033 from bacterial origin and 412 from eukaryotic origin (Table S5a and S5b). The branch including the Uniprot sequences of Eukaryote origin is displayed in blue. The kingdom origin of the sequence is indicated by the inner crown at the outside of the three. The data set covers 144 taxonomic orders as indicated by the outside crown. The Groups of the yeast sequences as defined in Fig. 3 are zoomed out and their position on the tree is denoted with dashed rectangle. The tree is build based on a Muscle muti sequence alignment. A zoomable tree is provided in Fig. S1. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

species abbreviation. This nomenclature will be used consistently throughout the remainder of this study (Table 2).

3.2. Characterisation of novel α -acetolactate decarboxylases from Saccharomycotina yeasts via heterologous expression in Saccharomyces pastorianus

To investigate the functional properties of eukaryotic α-acetolactate decarboxylases, seven candidate genes were selected based on their phylogenetic grouping and putative evolutionary origins (Table 2). Two genes from Group 3 were selected: *ACD1* from *Lypomyces arxi* (2a_LaNRRLY17921 222, *LaACD1*) and *ACD1* from *Dipodascus fermentans* (1a_DfCiCC 1368 8094, *DfACD1*). Additionally, two Group 4 genes were chosen: *ACD1* from *Wickerhamiella domercqiae* (16b_WdNRRL Y-6692_7, $WdACD1_{NRRLY-6692}$) and *ACD1* from *W. versatilis* (17f_WvJCM 5958_2, $WvACD1_{JCM5958}$). The proteins encoded by these four genes clustered within the eukaryotic Acd clade (Fig. 3). Two additional genes were selected due to their sequence divergence from the core eukaryotic group: a second *ACD* gene from *W. versatilis* (17e_WvJCM 5958_3, $WvACD2_{JCM5958}$) belonging to Group 2 and an unique *ACD1* gene from *W. azyma* (14a_WaPYCC 8333_20, *WaACD1*), forming Group 1 (Figs. 2 and 3). The seventh gene was the reference prokaryotic *BbaldB* from *Brevibacillus brevis*, previously characterized (Diderichsen et al., 1990; Svendsen et al., 1989).

The *in vivo* activity of these Saccharomycotina α-acetolactate decarboxylases was assessed through heterologous expression in lager brewing strain *S. pastorianus* CBS 1513, a member of the Group I (Saaz). Activity was estimated based on their ability to reduce vicinal diketones, namely diacetyl and 2,3-pentanedione, during the fermentation. These eukaryotic Acd were also benchmarked to the well-known prokaryotic α-acetolactate decarboxylase from *Brevibacillus brevis* (*Bb*AldB). All genes were codon optimized for *S. cerevisiae*, integrated into the chromosomal site ScYCR087C on *Se-Sc*CHRIII of strain CBS 1513 and expressed under the control of the strong constitutive *ScTDH3* promoter (Bennis et al., 2023). Fermentations were conducted in septum flasks using whole malt wort (5.7 °P). Growth, sugar consumption, ethanol production, and vicinal diketone (diacetyl and 2,3-pentanedione) concentrations were monitored throughout. Recombinant strains exhibited similar profiles to the parent strain, with no significant differences in sugar consumption or ethanol production at fermentation end ($p > 0.05$) (Fig. 4A and B). As expected, expression of *BbaldB* (strain IMI609) significantly reduced diacetyl production over 10-fold lower at peak levels and 6-fold lower after 9 days, compared to the parent strain CBS 1513 (Fig. 4C). Notably, strains expressing *LaACD1,* $WdACD1_{NRRLY-6692}$ and $WvACD2_{JCM5958}$ resulted in even lower final diacetyl levels than IMI609 ($p < 0.05$), while other strains achieved similar reductions with a final diacetyl of 14.5–5 μg L^{-1}, $p > 0.05$) (Fig. 4C). All recombinant strains showed significantly reduced final diacetyl concentrations compared to the parent strain. Diacetyl clearance kinetics also varied. Using 20 μg L^{-1} as a threshold, IMI611 (*LaACD1*) and IMI631 ($WdACD1_{NRRLY-6692}$) achieved this level after just one day. IMI610 ($WvACD2_{JCM5958}$) required two days, similar to *BbaldB*-expressing IMI609. Strains IMI633 (*DfACD1*), IMI630 (*WaACD1*), and IMI632 ($WvACD1_{JCM5958}$) exhibited higher diacetyl peaks, similar in timing to CBS 1513 but with 10-fold lower amplitude, and took at least five days to drop below 20 μg L^{-1} (Fig. 4C), suggesting reduced catalytic efficiency. Still, all engineered strains outperformed the parental CBS 1513, which peaked at 1.19 ± 0.06 mg L^{-1} diacetyl and failed to reduce it below 20 μg L^{-1} within 9 days (Fig. 4C). In line with prior studies (Blomqvist et al., 1991; Suihko et al., 1990), expression of *ACD* genes also reduced 2,3-pentanedione formation. All recombinant strains showed up to 28-fold lower peaks and remained well below the sensory threshold (900–1000 μg L^{-1}) (Fig. 4D).

To confirm broader applicability, *BbaldB*, $WvACD2_{JCM5958}$, and *LaACD1* were expressed in a Frohberg (Group II)-type lager strain (*S. pastorianus* CBS 1483). As observed in CBS 1513, these CBS 1483-derived strains (IMI483 (*BbaldB*), IMI603 ($WvACD2_{JCM5958}$), IMI604 (*LaACD1*)) also showed significant reductions in both diacetyl and 2,3-pentanedione, effectively eliminating the need for extended lagering (Fig. S2).

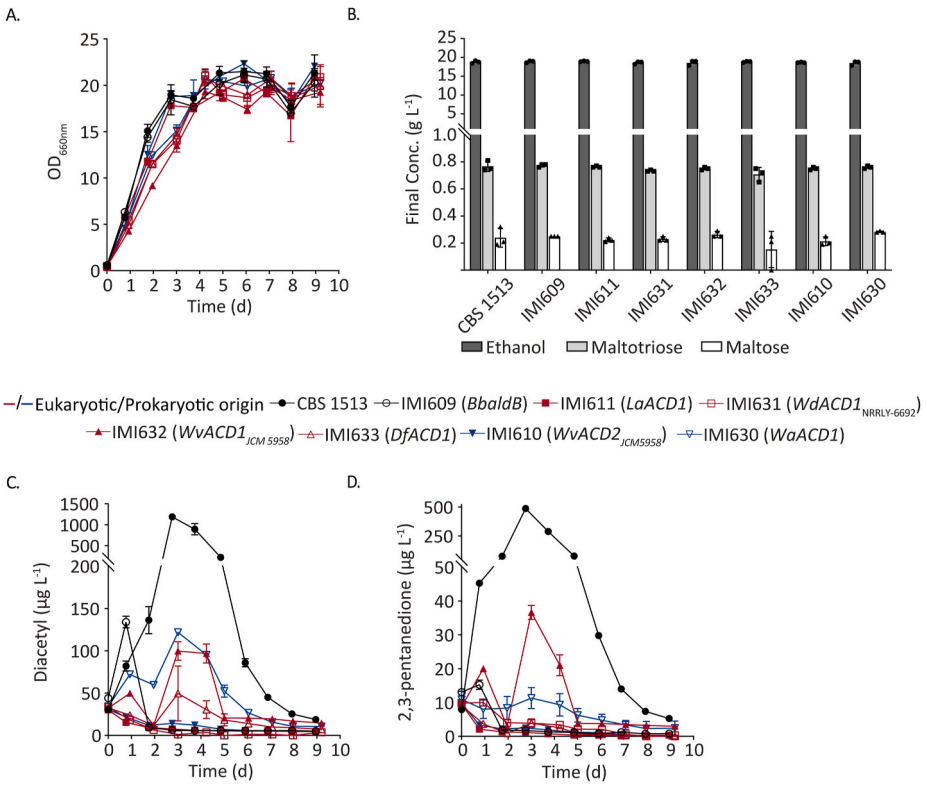

Fig. 4. Characterization of yeast Acd1 heterologously expressed in CBS 1513 in septum flasks. Strains were cultivated in whole malt wort (5.7°P) at 12 °C in septum flasks. A) Growth profile indicated by optical density (OD_{660nm}) over time. B) Final concentrations of ethanol and residual sugars at the end of fermentation (t = 9 days). C) Extracellular diacetyl concentration over time. D) Extracellular 2,3-pentanedione concentration over time. The values represent averages ± mean deviations of data obtained from independent triplicate cultures.

3.3. Fermentation performance of recombinant S. pastorianus expressing eukaryotic α-acetolactate decarboxylase in E.B.C tall tubes

Based on septum flask results, four strains, IMI611 (*LaACD1*), IMI631 ($WdACD1_{NRRLY-6692}$), IMI610 ($WvACD2_{JCM5958}$), and IMI609 (*BbaldB*) alongside the parent CBS 1513, were characterized under lager brewing relevant conditions in 2.25 L E.B.C. tall tubes with 17 °P whole malt wort at 12 °C. Fermentations were monitored for diacetyl and 2,3-pentanedione levels, sugar consumption, wort attenuation, and the production of ethanol, glycerol, higher alcohols, and esters. All recombinant strains showed slightly delayed sugar consumption and ethanol production compared to CBS 1513 (Fig. 5A). However, after 14 days, final ethanol levels and residual sugars for IMI611, IMI631, and IMI610 were comparable to the parent strain ($p > 0.05$). IMI609 (*BbaldB*), however, had significantly higher residual maltose (11.29 ± 7.90 g L^{-1}, $p < 0.05$) and lower final ethanol concentration (61.80 ± 4.71 g L^{-1}, $p < 0.05$) compared to CBS 1513 (2.32 ± 0.01 g L^{-1} and 68.65 ± 0.00 g L^{-1}, respectively). Despite the higher wort gravity, recombinant strains consistently maintained low diacetyl concentrations. After just one day, diacetyl levels dropped below 40 µg L^{-1}, whereas the parental strain reached 6.03 ± 0.27 mg L^{-1} on day four (Fig. 5B). All recombinant strains completed fermentation within 15 days (Fig. 5A) and finished with a diacetyl concentration lower than 10 µg L^{-1} and a 2,3-pentanedione concentration below 5 µg L^{-1} (Fig. 5B and C). CBS 1513, even after an extended incubation, only reduced diacetyl to ~120 µg L^{-1}, still six times above the sensory threshold. To estimate potential sensory impact in finished beer, diacetyl and 2,3-pentanedione concentrations on day 15 were normalized to 5 % ABV (39.45 g L^{-1} ethanol) (Fig. 5E and F). All *ACD1*-expressing strains produced beers with diacetyl levels well below the lowest documented sensory threshold (17 µg L^{-1}) (Meilgaard, 1982), with no significant differences between them ($p > 0.05$). These findings confirm that novel Acd1 from Saccharomycotina yeasts are as effective as *B. brevis* AldB in preventing vicinal diketones formation during lager fermentation (Fig. 5 and Fig. S3). In the meantime, the engineered strains (IMI609-611 and IM630-631) showed limited variations in other flavour-relevant compounds, such as higher alcohols and esters. Significant reduction were observed for isoamyl alcohol and ethyl esters, of which were present at concentrations up to 20 % lower in the green beers produced by the engineered *S. pastorianus* strains compared to CBS 1513 (Fig. 6). In contrast ethyl esters, ethyl butyrate and ethyl hexanoate were present in higher concentration again the difference between the reference CBS 1513 and the engineered strains did not exceed 20 % (Fig. 6C). As previously reported, the expression of an α-acetolactate decarboxylase does not impact the positive flavour note of the beverage while significantly reducing off-flavours.

3.4. Genome analysis of recombinant S. pastorianus strains

Although local genotyping of the engineered strains confirmed the integration of the α-acetolactate decarboxylase gene at the targeted locus, previous studies have shown that electroporation-based transformations can be mutagenic. In particular, they can induce changes in chromosome copy number, especially in aneuploid *S. pastorianus* strains (Gorter de Vries et al., 2020). Whole-genome sequencing of the engineered strains IMI609 (*BbaldB*), IMI610 ($WvACD2_{JCM5958}$), IMI611 (*LaACD1*), IMI630 (*WaACD1*), IMI631 ($WdACD1_{NRRLY-6692}$), IMI632 ($WvACD1_{JCM5958}$), and IMI633 (*DfACD1*) revealed subtle changes in chromosome copy number (Table S4). For instance, IMI611 (*LaACD1*) carried an extra copy of chromosome SeXII, IMI631 ($WdACD1_{NRRLY-6692}$) had an additional copy of chromosome *Se*ScIII, and IMI632 exhibited an extra copy of chromosome *Se*XVI. Moreover, all strains, except IMI631 ($WdACD1_{NRRLY-6692}$) and IMI633 (*DfACD1*), displayed segmental copy number variation near the insertion site on the

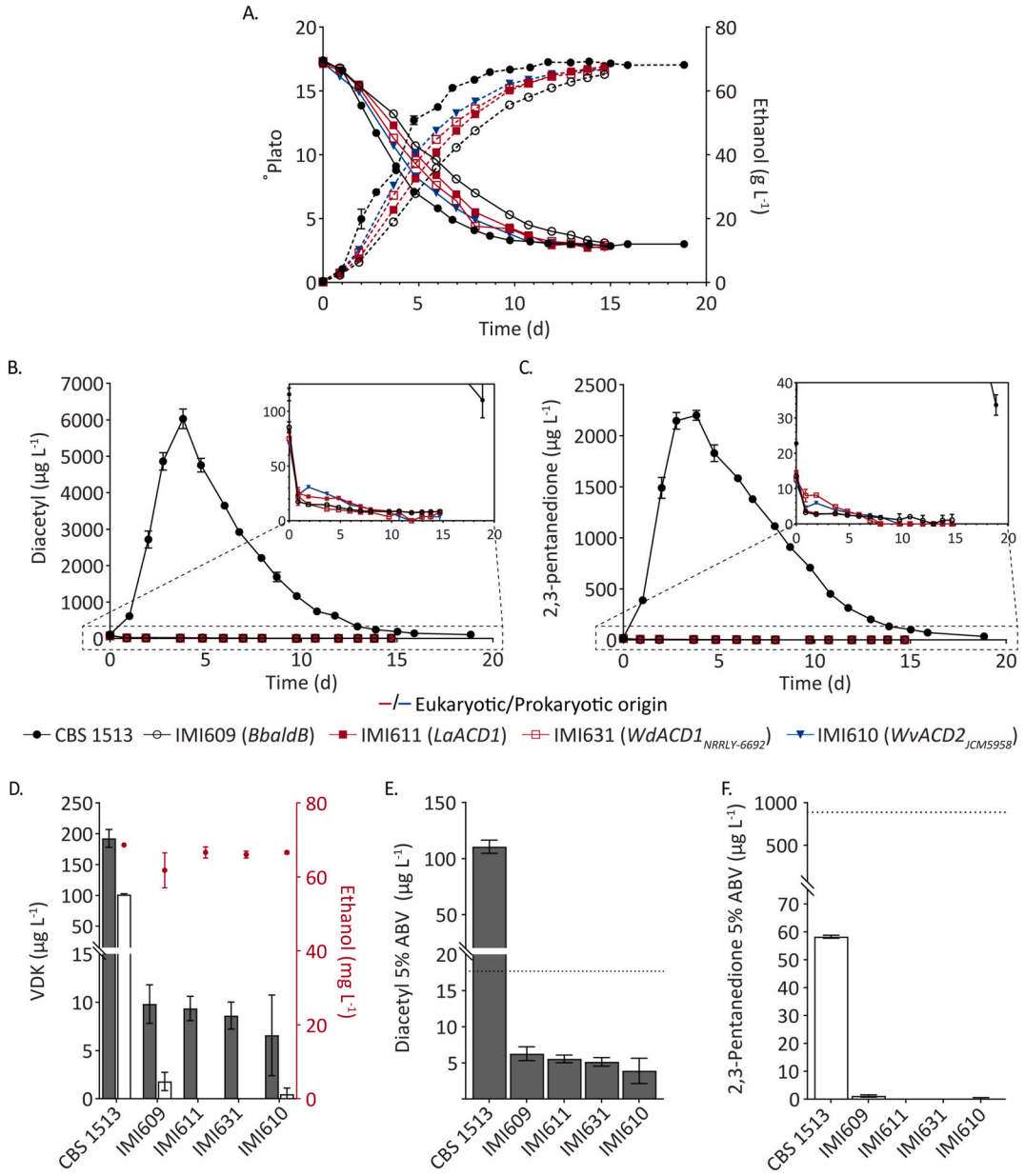

Fig. 5. Characterization of yeast Acd1 heterologously expressed in CBS 1513 in E.B.C. tall tubes. Strains were cultivated in whole malt wort (17°P) at 12 °C in E. B. C. tall tubes. A) Degrees plato (solid lines) and ethanol profile (dotted lines) over time. B) Extracellular diacetyl concentration over time. C) Extracellular 2,3-pentanedione concentrations over time. The data for strains IMI609 (*BbAldB*), IMI610 (*WvACD2*$_{JCM5958}$), IMI611 (*LaACD1*) and IMI631, (*WdACD1*$_{NRRLY-6692}$) presented in panels in A, B, and C are derived from single fermentation and display averages ± mean deviations of technical duplicates. The data derived from the biological duplicate cultures can be found in Fig. S3. The data for the reference *S. pastorianus* strain CBS 1513 shown in panel A,B and C are average ± mean from two independent biological duplicate fermentations. D) Concentration of diacetyl (grey bars), 2,3-pentanedione (white bars) and ethanol (red dots) on the 15th day of fermentations. E) Concentration of diacetyl on the 15th day of fermentation corrected for 5 % ABV. F) Concentration of 2,3-pentanedione on the 15th day of fermentation corrected for 5 % ABV. The dotted line in E and F indicates the sensory threshold of the vicinal diketone. The values represent averages ± mean deviations of the data obtained on the 15th fermentation day from two independent fermentations. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

right arm of chromosome *Sc*III and *SeSc*III. An approximately 100 kb region, spanning positions 210,000 to 310,000, showed variable copy numbers: four in CBS 1513 and IMI633 (*DfACD1*), five in IMI609 (*BbaldB*), and six in IMI611 (*LaACD1*) (Fig. 7). Copy number estimates of the *ACD* genes correlated with the copy number of CHRIII in each strain, suggesting that during homology-directed repair, integration of the *ACD* expression cassettes led to either duplication or triplication events, resulting in chromosomes extended by 100 or 200 kb (Fig. 7). This implies that absolute comparisons between the engineered strains are not feasible due to genomic variation; therefore, only qualitative comparisons are appropriate.

4. Discussion

This study provides the first evidence of functional α-acetolactate decarboxylase activity encoded by genes from yeasts within the Saccharomycotina subphylum. While this enzyme activity has long been exploited in the brewing industry for their ability to reduce diacetyl through expression of bacterial genes such as those from *Brevibacillus brevis* (Blomqvist et al., 1991; Svendsen et al., 1989; Yamano et al.,

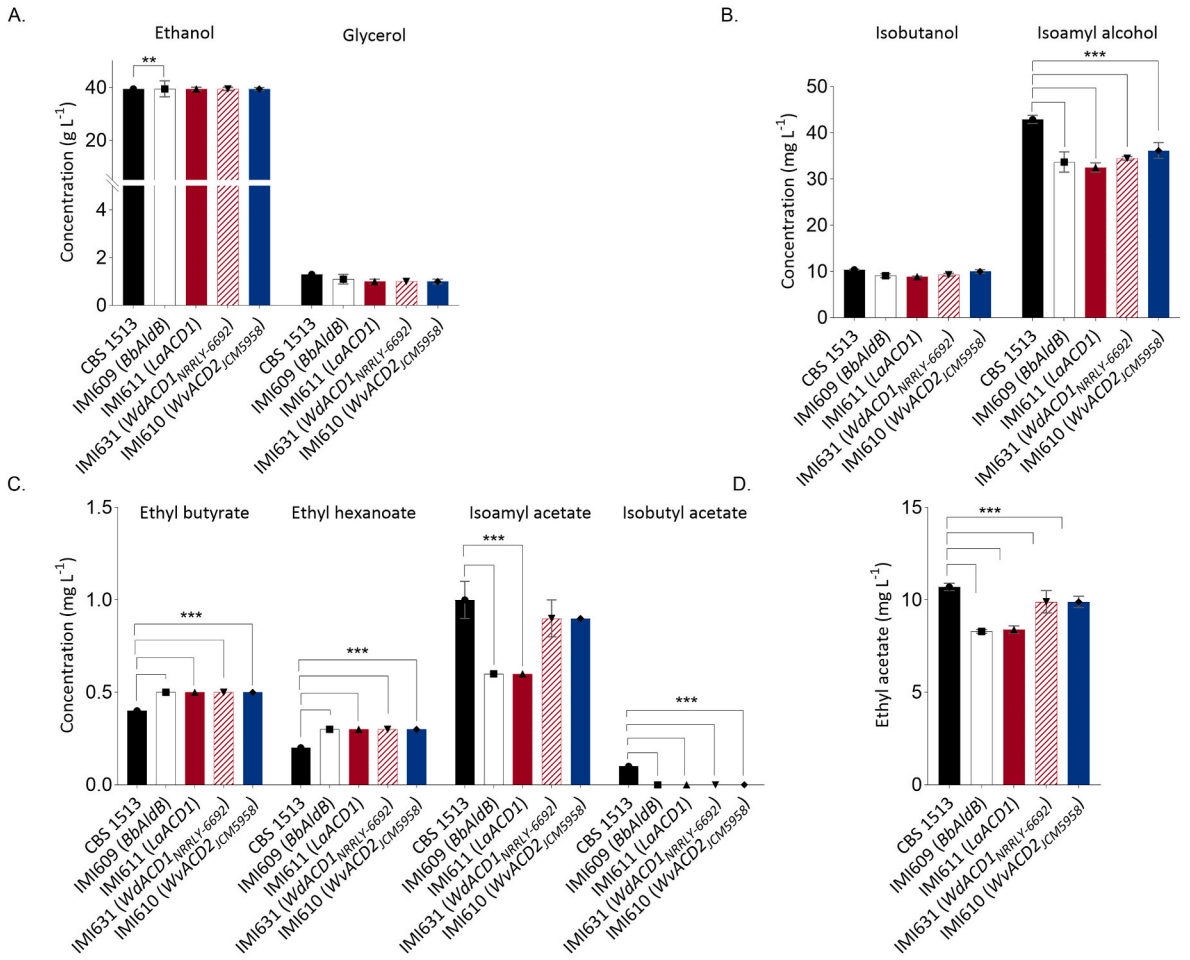

Fig. 6. Profile of flavouring higher alcohols and esters in whole malt wort fermentation of the reference *S. pastorianus* strain CBS 1513 and engineered *S. pastorianus* strains derived from CBS 1513 expressing heterologous Acd1. Strains were cultivated in whole malt wort (17 °P) at 12 °C in E. B. C. tall tubes. A) Concentration of ethanol and glycerol measured on the 15th day of the fermentation. B) Concentration of higher alcohols isobutanol and isoamyl alcohol measured on the 15th day of the fermentation. C) Concentration of ethyl esters (ethyl butyrate and ethyl hexanoate) and acetyl esters (isoamyl acetate and isobutyl acetate measured on the 15th day of the fermentation. D) Concentration of ethyl acetate measured on the 15th day of the fermentation.The values represent averages ± mean deviations of the data obtained from two independent fermentations.

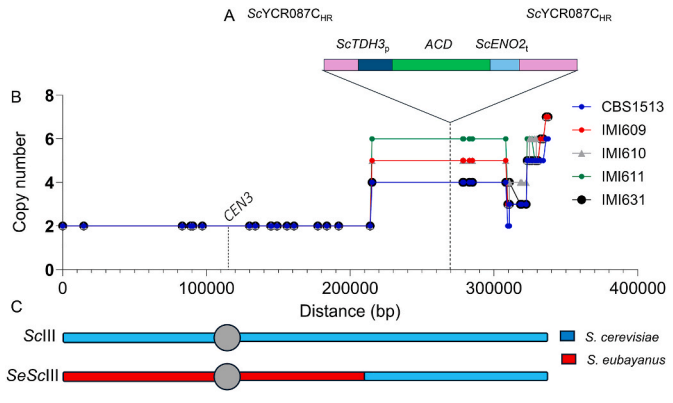

Fig. 7. Copy number of CHR *Sc*III and section including the end of the left arm of CHR *Se*ScIII in *S. pastorianus* engineered strains. A) Schematic representation of the *ACD* expression cassette integration at the ScYCR087C locus. B) Segmental copy number estimation of CHR *Sc*III in the *S. pastorianus* parental strain CBS 1513 and the α-acetolactate decarboxylase expressing strains IMI609 (*BbaldB*), IMI610 (*WvACD2*$_{JCM5958}$), IMI611 (*LaACD1*) and IMI631 (*WdACD1*$_{NRRLY-6692}$). C) Schematic representation of the two type of CHRIII structures found in the *S. pastorianus* strain CBS 1513 (Walther et al., 2014).

1994), their eukaryotic counterparts remained unexplored. Our findings and functional characterization of several yeast-derived α-acetolactate decarboxylase enzymes not only expand the known diversity of this enzyme class but also provides viable eukaryotic alternatives to prokaryotic α-acetolactate decarboxylase for industrial applications. Although this study did not include a comparative kinetic analysis of the different enzymes, owing to the complexity of measuring α-acetolactate decarboxylase activity. The use of heterologous expression in *Saccharomyces pastorianus* provided a practical alternative. This approach enabled a direct functional comparison between yeast-derived α-acetolactate decarboxylase and the well-characterized *B. brevis* enzyme (*Bb*AldB) in brewing context, specifically during wort fermentation and at low temperature. The measurement of α-acetolactate decarboxylase activity is challenging because α-acetolactate, the natural substrate, is not commercially available in its native form; it is typically supplied as esterified derivatives (e.g., ethyl 2-acetoxy-2-methylacetoacetate) to prevent spontaneous decarboxylation. Quantification requires saponification followed by α-acetolactate recovery under a nitrogen atmosphere, a labor-intensive process (Dulieu and Poncelet, 1999). Interestingly, while all expressed eukaryotic *ACD* genes significantly reduced diacetyl levels, variations in the rate of diacetyl clearance suggest difference in enzyme activity. These results align with previous observations indicating that even bacterial α-acetolactate decarboxylases exhibit variable catalytic properties depending on their origin and

expression conditions (Blomqvist et al., 1991; Yamano et al., 1994). The slower diacetyl clearance observed in strains expressing *DfACD1*, *WvACD1*$_{JCM5958}$, and *WaACD1* which may reflect differences in enzyme kinetics or stability, should be weighted by the difference in *ACD* genes copy number in each engineered strain, these factors warranting further investigation. Notably, some yeast α-acetolactate decarboxylases performed comparably or even better than their bacterial counterpart in reducing vicinal diketones, highlighting their potential for industrial deployment.

The yeast species harbouring α-acetolactate decarboxylase genes appear to be restricted to only three genera, *Dipodascus*, *Lipomyces*, and *Wickerhamiella*. all of which are phylogenetically positioned outside the core budding yeasts (Saccharomycetaceae). These genera are considered early-diverging lineages within the Saccharomycotina subphylum (Shen et al., 2018). This phylogenetic placement suggests that they may retain ancestral features lost in more derived clades. This hypothesis is partially supported by the identification of orthologous α-acetolactate decarboxylase in 27 different fungal orders (Fig. 3). Despite this broader distribution, α-acetolactate decarboxylases remain entirely uncharacterized in fungi beyond those studied here.

In addition to these fungal sequences, four other yeast α-acetolactate decarboxylase sequences were found to cluster outside the eukaryotic clade (Fig. 3) and exhibit higher similarity to bacterial sequences, suggesting an alternative evolutionary origins, such as horizontal gene transfer (HGT). HGT is commonly associated with adaptation to competitive ecological niches. *Wickerhamiella* (de Vega et al., 1970; Lachance et al., 2001; van der Walt and Liebenberg, 1973) and *Dipodascus* (Weijman, 1979) species are frequently isolated from environments such as flowers, fruits, and insects (e.g. particularly honeybees), while *Lipomyces* species are commonly found in soil and decaying plant matter (Gouliamova et al., 1998). These habitats are often rich in bacterial competitors and may therefore favour the acquisition of bacterial genes conferring selective metabolic advantages. In lactic acid bacteria such as *Lactococcus lactis*, α-acetolactate decarboxylase plays a dual role: (i) regulating valine and leucine biosynthesis by modulating α-acetolactate flux, and (ii) catalyzing the second step of the 2,3-butanediol pathway (Goupil-Feuillerat et al., 1997). In contrast, yeasts regulation of branched-chain amino acid biosynthesis differs significantly (Sze et al., 1992). It is likely that α-acetolactate decarboxylase provides these yeast a selective advantage in environments potentially rich in diacetyl, converting it to less toxin acetoin. Diacetyl has been reported to exert antimicrobial effects, particularly against *Escherichia coli* and *Listeria monocytogenes*, and is implicated in spoilage prevention (Cui et al., 2025). However, no comprehensive studies have examined its inhibitory effects on yeast growth.

Further evidence supporting an HGT event can be observed within the *Wickerhamiella* genus. Of the more than 50 known species (https://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?id=45787), 28 have been sequenced (Opulente et al., 2024) and were included in the search for *Bb*AldB orthologs. Remarkably, only two species (*W. versatilis* and *W. azyma*) possess a prokaryotic-type α-acetolactate decarboxylase. This patchy distribution within a single genus strongly supports the hypothesis of horizontal gene transfer. The *Wickerhamiella* genus is already known for extensive HGT, with some species acquiring up to 169 genes from bacteria (Shen et al., 2018; Gonçalves et al., 2020). Despite this, the transfer of a α-acetolactate decarboxylase gene had not been reported in *Wickerhamiella* species until now.

By maintaining diacetyl concentrations below sensory thresholds, the expression of α-acetolactate decarboxylase can significantly reduce or even eliminate the need for extended lagering periods, one of the major bottlenecks in lager beer production (Krogerus and Gibson, 2013a). Life Cycle Assessment (LCA) has been applied to evaluate the environmental impacts associated with each stage of beer production, from raw material acquisition (e.g., malt and hops) to brewing, packaging, distribution, and disposal. LCA identifies environmental "hotspots" including energy consumption, greenhouse gas (GHG) emissions, water usage, and waste generation. While fermentation and maturation stages are less energy-intensive than process stages such as wort boiling or packaging, they still contribute substantially to the overall environmental footprint (Talve, 2001). Key areas of impact of fermentation and maturation phase include: i) energy consumption (lager fermentation and maturation require substantial cooling capacity and prolonged storage time), ii) greenhouse gas (GHG) emissions (carbon dioxide (CO_2) generated during fermentation is typically vented unless specifically captured and reused (e.g., for carbonation); iii) water use and wastewater generation (cleaning of fermenters and maturation tanks consumes significant water and produces wastewater that often contains organic loads (e.g., residual sugars, yeast cells) requiring treatment). Though the exact environmental impact of each production stage varies by brewery size, technological infrastructure, and geographic context, it is estimated that fermentation and maturation contribute approximately 20–30 % of energy consumption, 10–20 % of GHG emissions, and 15–25 % of water usage in beer production (D'Ascenzo et al., 2024; De Marco et al., 2016; Salazar Tijerino et al., 2023). These figures highlight the significant sustainability gains that could result from reducing or eliminating the lagering phase.

This study demonstrates that genetically modified yeast strains can offer significant technical advantages in brewing, including accelerated fermentation, enhanced flavour profiles, and effective diacetyl control. The *Saccharomyces pastorianus* strain CBS 1513, used as a model in this study, is also known as "Unterhefe Nr. I" (bottom-fermenting yeast No. 1) and is considered the closest available strain to the one originally isolated by Emil Christian Hansen in 1883 (Hansen, 1883, 1908). CBS 1513 is notable for its exceptional ability to ferment maltotriose, one of the wort sugars that is not always efficiently consumed by lager yeasts (Magalhaes et al., 2016). However, despite this advantageous trait, CBS 1513 has lost appeal due to its high diacetyl production, which necessitates prolonged lagering. The expression of an α-acetolactate decarboxylase, as demonstrated in this study, would enable the exploitation of CBS 1513's efficient maltotriose fermentation while mitigating diacetyl accumulation. However, the widespread adoption of such a strain remains constrained by regulatory, commercial, and perceptual barriers. In Europe, genetically modified yeast strains are classified "novel organisms" and require pre-market approval under Regulation (EC) No 1829/2003 (Regulation (EC) No 1829/2003 of the European Parliament and of the Council of September 22, 2003 on genetically modified food and feed (Text with EEA relevance) October 18, 2003), resulting in lengthy and complex authorization processes. Consumer perception remains the principal limitation. Beer is often marketed as a natural, traditional, and artisanal product, an image that conflicts with the use of genetically modified yeast in the eyes of many consumers. Surveys consistently show strong consumer reluctance, particularly in Europe, toward GMO-derived food and beverages, even when the final product contains no trace of the modified organism (https://foodinsight.org/wp-content/uploads/2021/05/IFIC-2021-Food-and-Health-Survey.May-2021-1.pdf) (Vecchione et al., 2015). Despite this scepticism, several strains expressing α-acetolactate decarboxylase are already available from companies such as Omega Yeast (https://omegayeast.com/) and Berkeley Yeast (https://berkeleyyeast.com/), both of which are pioneering this approach commercially. Notably, these companies are based in North America, where regulatory frameworks and public acceptance of engineered yeasts are generally more permissive. Although non-GMO approaches, such as selective breeding, laboratory evolution (Brickwedde et al., 2017; Mans et al., 2018; Gibson et al., 2018), and hybridization (Krogerus et al., 2017; Gorter de Vries et al., 2019), remain options, these methods are time-consuming, costly, unpredictable, and often less efficient. This study uncovers a previously unrecognized enzymatic function in Saccharomycotina yeasts and demonstrates the efficacy of yeast-derived α-acetolactate decarboxylases in reducing diacetyl levels during lager fermentation. These findings not only broaden the known diversity of α-acetolactate decarboxylases but also provide a safe and efficient strategy to improve

process performance and sustainability in beer production.

CRediT authorship contribution statement

Maartje Spaans: Writing – original draft, Methodology, Investigation. **Leah S. Winkler:** Investigation. **Marcel A. van den Broek:** Visualization, Software, Methodology, Formal analysis, Data curation. **Jean-Marc G. Daran:** Writing – original draft, Project administration, Methodology, Funding acquisition, Conceptualization.

Funding

This work was performed within the Top consortia for Knowledge and Innovation (TKIs) AgriFood which was granted a PPP allowance from the Dutch Ministry of Economic Affairs and Climate Policy (Project ALBEyDO #PPS2204).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We thank Nicole X. Bennis, Ewout Knibbe, Ilja Westerlaken and Flip A. de Groot for their technical advices. We are thankful to Prof. JT. Pronk (Delft University of Technology), Dr. V.M. Boer and Mr T. Elink Schuurman (HEINEKEN Supply Chain B.V.) for their support during this project.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2025.104903.

Data availability

Data underlying this study are available at the 4TU Research Data repository (https://data.4tu.nl/)with the DOI: https://doi.org/10.4121/3fa366a0-20bd-4d7e-9243-78959527fb07.

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