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Identification of Sc-type *ILV6* as a target to reduce diacetyl formation in lager brewers' yeast

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ABSTRACT

Diacetyl causes an unwanted buttery off-flavor in lager beer. It is spontaneously generated from α -acetolactate, an intermediate of yeast's valine biosynthesis released during the main beer fermentation. Green lager beer has to undergo a maturation process lasting two to three weeks in order to reduce the diacetyl level below its taste-threshold. Therefore, a reduction of yeast's α -acetolactate/diacetyl formation without negatively affecting other brewing relevant traits has been a long-term demand of brewing industry. Previous attempts to reduce diacetyl production by either traditional approaches or rational genetic engineering had different shortcomings. Here, three lager yeast strains with marked differences in diacetyl production were studied with regard to gene copy numbers as well as mRNA abundances under conditions relevant to industrial brewing. Evaluation of data for the genes directly involved in the valine biosynthetic pathway revealed a low expression level of Sc-*ILV6* as a potential molecular determinant for low diacetyl formation. This hypothesis was verified by disrupting the two copies of Sc-*ILV6* in a commercially used lager brewers' yeast strain, which resulted in 65% reduction of diacetyl concentration in green beer. The Sc-*ILV6* deletions did not have any perceptible impact on beer taste. To our knowledge, this has been the first study exploiting natural diversity of lager brewers' yeast strains for strain optimization.

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1. Introduction

Although beer brewing is a well-established process brewers are still interested in optimizing their yeast strains, particularly with regard to beer stability, the development of novel flavors and economics of the brewing process (Donalies et al., 2008; Saerens et al., 2010). In general, there are two types of brewer's yeast, i.e. top- and bottom-fermenting strains used to produce ale and lager beer, respectively. Lager brews account for the major part (90%) of the world's beer production and most research has focused on lager yeast (Kodama et al., 2006). Such strains are aneuploid genetic hybrids, which have been originally denoted as *Saccharomyces carlsbergensis* and nowadays classified as *Saccharomyces pastorianus* (Hansen and Kielland-Brandt, 2003; Kodama et al., 2006; Vaughan-Martini and Kurtzman, 1985). They contain chromosomal sequences originating from *Saccharomyces cerevisiae*

and from another *Saccharomyces* species, possibly represented by *Saccharomyces bayanus*. Due to this hybrid nature of lager brewers' yeast, the majority of ORFs is present in two homologous versions (orthologs), which are referred to as Sc-genes and Sb-genes. In addition, lager brewers' yeast strains contain ORFs, which are not present in *S. cerevisiae* at all (Nakao et al., 2009; Yoshida et al., 2007).

Diacetyl has a butter-like flavor and is particularly undesirable in lager beers. Its concentration in green beer (beer after main fermentation) is usually far above diacetyl's taste threshold in lager beer, which is 0.15 ppm or even lower (Saison et al., 2009). Lager beer has to be stored for 2–3 weeks at a temperature close to the freezing point until diacetyl concentration has declined below its taste threshold. This maturation phase requires storage capacities and controls the output of beer from a brewery.

Diacetyl (2,3-butanedione) is a vicinal diketone and formed via a non-enzymatic decarboxylation from α -acetolactate outside the cell (Haukeli and Lie, 1978). The latter compound is an intermediate of the valine biosynthetic pathway. Diacetyl is reabsorbed by the yeast cell and converted to acetoin and subsequently to 2,3-butanediol by

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the action of 2,3-butanediol dehydrogenase and other not fully characterized ketoreductase(s) (Ehsani et al., 2009; Gonzalez et al., 2000). Compared to diacetyl, 2,3-butanediol has a much higher taste threshold. The diacetyl concentration during the different stages of a brewing fermentation is the result of several superimposed processes, i.e. the regulation of valine uptake by available free amino nitrogen (FAN) present in wort, the feed-back inhibition of valine biosynthetic pathway by intracellular valine, the factors that influence the chemical conversion of α -acetolactate into diacetyl and the enzymatic reduction of diacetyl into acetoin and 2,3-butanediol. In a usual beer fermentation with sufficient FAN, diacetyl forms a peak at about 48 h of fermentation when the rapid uptake of valine and other B-type amino acids begins (Petersen et al., 2004).

One approach to prevent the formation of diacetyl has been the addition of the enzyme α -acetolactate decarboxylase (ALDC) to green beer. This enzyme catalyzes the direct conversion of α -acetolactate to acetoin, thereby preventing diacetyl formation. The addition of *Enterobacter aerogenes* ALDC to green beer led to a decrease in vicinal diketone levels below the taste-threshold after 24 h at 10 °C (Godtfredsen et al., 1987). Genes encoding ALDC from different bacteria, e.g. *E. aerogenes*, *Klebsiella terrigena*, *Lactococcus lactis* and *Acetobacter aceti*, were also expressed in yeast using either episomal plasmids or genomic integrations (Blomqvist et al., 1991; Fujii et al., 1990; Goelling and Stahl, 1988; Sone et al., 1988; Sone et al., 1987; Yamano et al., 1994).

As consumer acceptance for genetically modified brewers' yeast containing bacterial genes has been extremely low, other strategies to reduce diacetyl have focused on rationally engineering yeast's native valine biosynthetic pathway. One obvious target has been the formation of the precursor α -acetolactate. Different attempts eliminating or reducing the activity of acetohydroxyacid synthase (AHAS, acetolactate synthase) have been published (Gjermansen et al., 1988; Kiellandt-Brandt et al., 1990; Liu et al., 2004; Vakeria et al., 1991; Zhang et al., 2008). A second approach has been an enhanced conversion of its precursor α -acetolactate to valine. To this end, overexpression of *ILV3* encoding dihydroxyacid reductase and/or *ILV5* encoding reductoisomerase was performed (Goossens et al., 1993, 1987; Mithieux and Weiss, 1995; Omura, 2008; Villanueva et al., 1990). For more detailed reviews regarding genetic approaches for diacetyl reduction in beer the reader is referred to Donalies et al. (2008), Nevoigt (2008) and references cited therein.

Virtually all previous approaches to reduce diacetyl formation have been based on rational engineering. The knowledge concerning metabolic pathways, enzymes and their kinetics used to generate a rational engineering strategy originates for the most part from studies with *S. cerevisiae* (particularly from laboratory strains under laboratory conditions) and does not allow for the specific constraints resulting from industrial brewing conditions and strains. Due to these issues, results obtained in laboratory strains/conditions are often not transferable to industrial conditions. An attractive alternative to engineer industrially relevant

traits is to start from an interesting phenotype possessed under industrial conditions, identify its molecular rationale and transfer it to the industrial host strain. This strategy referred to as inverse engineering (Bailey et al., 2002) requires phenotypic diversity. Here, we describe the analysis of three lager brewers' yeast strains with significant differences in diacetyl formation as well as the identification and verification of low *Sc-ILV6* expression level as one reason for low diacetyl production.

2. Materials and methods

2.1. Microbial strains, media and growth conditions

The *E. coli* strain DH5 α TM (Invitrogen Corp., Carlsbad) was used for amplification of plasmids. *E. coli* cultivation, transformation and plasmid isolation were carried out using standard techniques (Sambrook et al., 1989). Yeast strains used in this study are listed in Table 1. Apart from brewers' wort fermentations (see below), yeast was grown in Erlenmeyer flasks on a rotary shaker at 170 rpm in YEPD medium (1% yeast extract, 2% peptone and 2% glucose) at 30 °C or on YEPD agar plates (YEPD medium plus 1.5% agar).

Based on available information about diacetyl production characteristics, the following three lager brewers' strains from the strain collection of the "Institut für Gärungsgewerbe Berlin" were chosen for our study: Sa-06165, Sa-06136 and Sa-06168 (Table 1). For simplicity, the strains were tagged with the codes MD (Medium Diacetyl), HD (High Diacetyl) and LD (Low Diacetyl) throughout the study. Strain MD is a commercially used production strain for lager beer brewing. Strain HD is a former production strain with slightly higher diacetyl production compared to strain MD. In fact, strain HD is the origin of strain MD; the latter was obtained by single cell isolation from a culture of strain HD. Therefore, high similarity between strains HD and MD was expected. Finally, strain LD is of unknown origin but was previously selected for a very low level of diacetyl production (J. Methner, personal communication).

2.2. Determination of cell density

During yeast cultivations in YEPD, cell density was recorded by measuring optical density using a spectrophotometer at 600 nm (OD₆₀₀). As brewers' wort contains particulate matter falsifying measurements of optical density, all cell densities in this medium were determined using a Thoma counting chamber.

2.3. Brewers' wort fermentations

Typical Pilsener wort with an original gravity between 11.1 and 11.5°P was kindly provided by a German brewery or by the Chair of Brewing Science of Berlin University of technology. Different batches of wort were used throughout the study. For

Table 1
Yeast strains used in this study.

Strain	Code	Genotype	Source or reference
Sa-06136	HD	Lager brewers' yeast	Yeast strain collection "Institut für Gärungsgewerbe Berlin"
Sa-06168	LD	Lager brewers' yeast Selected for very low diacetyl production	Yeast strain collection "Institut für Gärungsgewerbe Berlin"
Sa-06165	MD	Lager brewers' yeast derived from strain Sc-06136 via single cell isolation	Yeast strain collection "Institut für Gärungsgewerbe Berlin"
Sa-06165 <i>Scilv6Δ</i>	<i>Sc-ilv6Δ</i>	<i>Sc-ILV6/Sc-ilv6Δ:: <i>loxP-KanMX-loxP</i></i>	This study
Sa-06165 <i>Sc-ilv6Δ/<i>Scilv6Δ</i></i>	<i>Sc-ilv6Δ/Δ</i>	<i>Sc-ilv6Δ::<i>loxP-KanMX-loxP/Sc-ilv6Δ:: <i>loxP-ble</i>^r-<i>loxP</i></i></i>	This study

main fermentations in 3-l or 25-l scale, 200 ml wort was inoculated with yeast from agar plates and cultures were incubated at room temperature with shaking. The volume was stepwise increased by adding fresh brewers' wort to the culture as soon as a cell density of 1×10^8 cells/ml was reached. During each volume increase, the cell density was adjusted between 5×10^6 and 1×10^7 cells/ml. Using this feeding strategy, the preculture was always kept in the exponential growth phase. Before inoculating the main fermentation, the preculture was incubated without shaking at 12 °C for 24 h in order to adapt the yeast to a lower temperature. In order to inoculate the main fermentations in 3 l scale, the cells were harvested by centrifugation (5 min at 4000 rpm, Sorvall, rotor GS-3) and used in order to inoculate fresh wort at a cell density of 5×10^6 cells/ml. Three liter scale fermentations were performed in a 4.8-l glass bioreactors (Jenaer Glas AG, Jana, Germany) with stirring at 50 rpm at 10 °C. The bioreactors were closed with airlocks for elimination of oxygen but allowing the release of gases. For 25-l scale main fermentations, the last preculture (5 l) was transferred into 30-l scale tanks and filled up with fresh wort to a volume of 25 l resulting in a cell density of 1.6×10^7 cells/ml. Main fermentations were carried out at 11 °C.

Cell density, apparent extract of wort and diacetyl concentration was recorded throughout the main fermentation. Fermentations were finished when wort apparent extract had reached a value between 2.8% and 3%.

2.4. DNA microarrays

The arrays used in this study for both comparative genome hybridization and transcriptome analysis were customized lager brewing yeast arrays kindly provided by Suntory Ltd. (Affymetrix GeneChip® Custom Express™) containing 22,977 probe sets able to distinguish between *S. cerevisiae* (Sc)-type genes and *S. bayanus* (Sb)-type genes from lager brewers' yeast (Horinouchi et al., 2010).

2.5. Microarray-based comparative genome hybridization (CGH)

Genomic DNA extraction and sample preparation was carried out according to a method described by Winzeler et al. (2003) with slight modifications. Yeast culture grown in YEPD (50 ml) was harvested at an OD₆₀₀ of 1.5. Genomic DNA (10 µg) was fragmented using 0.15 units DNase I (Gibco BLR, PCR grade) in $1 \times$ One-Phor-All buffer (Pharmacia) supplemented with 1.5 mM CoCl₂ for 3 min at 37 °C. DNase I reaction was inactivated by heating the sample at 95 °C for 15 min. Fragmented DNA was labeled with 1 nmol Biotin-N6-ddATP (NEL) using 25 units terminal transferase (Roche) at 37 °C for 1 h. Labeled DNA fragments were dissolved in 200 µl hybridization solution containing 50 pM Control Oligonucleotide B2 (Affymetrix), $1 \times$ Eukaryotic Hybridization Controls (Affymetrix), 20 µg herring sperm DNA (Promega), $6 \times$ SSPE (0.9 M NaCl, 60 mM NaH₂PO₄, 6 mM EDTA) (NIPPON-GENE) and 0.005% Triton-X (SIGMA). After 10 min incubation at 100 °C, the hybridization solution was transferred on ice for a few minutes and afterwards hybridized to the DNA microarray. Genomic DNA of each lager yeast strain was hybridized to one single array. Hybridization, washing, staining and scanning of the arrays was carried out as described in Affymetrix users' manual (Affymetrix, 2004). The hybridization intensities and call for absence and presence of genes were computed using Affymetrix GeneChip® Operating Software (GCOS) v 1.0. For each pairwise comparison, ratio of signal intensity and corresponding *p*-value for differences in signal intensity was calculated for every probe set. A sequence was designated as "increased" if the calculated change *p*-value was ≤ 0.002 and as "decreased" if the

change *p*-value was ≥ 0.998 (Affymetrix Inc.: Statistical algorithms description document, http://www.affymetrix.com/support/technical/whitepapers/sadd_whitepaper.pdf, 2002).

2.6. Microarray-based comparative transcriptome analysis

For RNA isolation, cells were harvested from the 25-l main fermentation when the apparent extract reached a value of 8%. Roughly 20 ml of culture corresponding to 240 mg yeast wet weight was harvested in triplicate for each strain. Cell sampling was performed as previously described (Piper et al., 2002) with slight changes. After thawing, the sample was centrifuged at 3000g (5000 rpm, Sorvall rotor SS-34) at 0 °C for 4 min and resuspended in 1.8 ml AE-cold buffer (50 mM sodium acetate, 10 mM EDTA, pH 5.0). The content of the two tubes was pooled and afterwards aliquoted into 10 Eppendorf tubes, 400 µl each. RNA extraction was carried out using the hot phenol method (Schmitt et al., 1990). The resulting RNA samples were treated with DNase I (Amersham) before array hybridization. Sample preparation, hybridization, washing and scanning of the arrays were performed following Affymetrix users' manual (Affymetrix, 2004). Array hybridizations were carried out with three technical replicates, i.e. three independent but parallel RNA isolations for each strain. Detection of signal intensities of microarrays was carried out using Affymetrix Gene Chip Analysis Basic System and GCOS v 1.0. Every transcript was flagged as absent "A", present "P" or marginal present "M" based on the detection *p*-value calculated by Detection Algorithm with default parameter in GCOS. Subsequently, the data was adjusted by quantile normalization (Bolstad et al., 2003). For every transcript in each pairwise comparison, average logged fold-changes were calculated. The significance of differential expression was assessed using a Bayesian t-test as implemented in the Goldenspike R-package (Baldi and Long, 2001; Choe et al., 2005). The derived *p*-values were adjusted for multiple testing and converted to false discovery rates (FDR) applying the Benjamini-Hochberg procedure. As significant threshold for the pairwise comparisons, a false discovery rate of 0.001 was chosen.

2.7. PCR amplification of the *Sc-ILV6* deletion cassettes

The disruption cassettes *loxP-KanMX-loxP* and *loxP-ble^r-loxP* were amplified from plasmids pUG6 and pUG66 (Guedener et al., 2002) using the same primer pair P1/P2 (Table 2). The 45 nt at the 5' end of primer P1 are homologous to the sequence upstream of the *Sc-ILV6* start codon while the 45 nt at the 5' end of P2 primer correspond to the sequence from position 552 to 596 of the *Sc-ILV6* coding sequence. The PCR conditions were as follows: (i) 95 °C for 2 min; (ii) 25 cycles: 94 °C for 45 s, 57 °C for 45 s and elongation (see below) at 72 °C; (iii) a final step for 10 min at 72 °C. Elongation times for the amplification of the *loxP-KanMX-loxP* and *loxP-ble^r-loxP* cassettes were 2 min and 1.5 min, respectively.

2.8. Disruption of two copies of *Sc-ILV6* in lager brewers' yeast

Brewers' yeast transformation was performed using the lithium acetate/PEG method (Gietz and Woods, 2002) using 1 µg of PCR product comprising the disruption cassette. Before transformation, yeast was grown in 200 ml YEPD medium at 30 °C to an OD₆₀₀ of 0.7 and cells were harvested by centrifugation (2 min at 5000 rpm, Sorvall, rotor GSA). After transformation, the transformation mixture was added to 5 ml YEPD and incubated overnight at 30 °C with shaking (170 rpm). Afterwards, cells were washed with 0.85% NaCl and spread onto YEPD agar plus

Table 2
PCR primers used.

Name	Oligonucleotides (5'–3')
P1	TACAGAATCTTTAGAACATCTGAGCTCACTAACCCAGTCTTTCTAccgccagctgaagcttcg ^a
P2	ATTTGGCGACCAATTTCTGGGAGTCAGCGGCCAGCAITGGTGcatagggcactagtggatc ^a
P3	ATATGGAAGTACATAGTTCC
P4	TTCGGCGACCAATTTCTG
P5	TAAGTCACATACGTAGTTTG
P6	TCGGCAACTAACTCGTTG

^a At the 3' end, the forward P1 and reverse P2 primers, respectively, contained 18 bp and 20 bp homologous to plasmid pUG6 and/or pUG66. At the 5' ends, the two primers contained 45 bp homologous to the sequence of Sc-ILV6.

Geneticin G418 (17.5 µg/ml) and/or phleomycin (17.5 µg/ml) depending on the disruption cassette.

2.9. PCR diagnostics of Sc-ILV6 single and double deletion

Genomic DNA was isolated from brewers' yeast transformants following the method of Hoffman and Winston (1987). To verify the Sc-ILV6 deletion, primers P3 and P4 (Table 2) were used. Primer P3 anneals 49 bp upstream of Sc-ILV6 ATG and primer P4 anneals between position 577 and 594 of the Sc-ILV6 coding sequence. Primers P5 and P6 were used to verify the presence of the Sb-ILV6 ORF. Primer P5 was homologous to the sequence located 46 bp upstream of Sb-ILV6 ATG. Primer P6 is homologous to the sequence between positions 576 and 593 of the Sb-ILV6 coding sequence. The PCR conditions were as follows: (i) 95 °C for 2 min; (ii) 25 cycles of 94 °C for 45 s, 50 °C for 45 s and 72 °C for 2 min; (iii) a final step for 10 min at 72 °C.

2.10. Determination of in vitro AHAS activity

Yeast strains were pre-cultivated in 20 ml brewers' wort by shaking at 24 °C for 24 h. Fresh wort (90 ml) was then inoculated with the pre-culture adjusting a cell concentration of 1×10^7 cells/ml. Fermentations were performed in 100 ml Schott bottles closed with airlocks at 12 °C until the apparent extract was reduced to a value between 8.7 and 8.3. Cells were harvested by centrifugation for 5 min at 5000 rpm (Sorvall, rotor GSA) at 4 °C and washed twice with sterile distilled water. Next, cells were resuspended in 1 ml ice-cold buffer (0.1 M Tris-HCl pH 7.5, 0.1 M NaCl and 0.1 M EDTA) supplemented with 2 mM PMSF. Cells were permeabilized by adding 100 µl chloroform and vortexed for 30 s. Samples were centrifuged at 6000 rpm (Biofuge Fresco, Heraeus, rotor 3325B) for 5 min 4 °C and the supernatant was removed. Permeated cells were resuspended in the same buffer and were placed at 4 °C. AHAS assay was performed within 2 h after sample preparation.

In vitro AHAS activity was measured based on its ability to convert pyruvate into α -acetolactate as described by Byrne and Meacock (2001) with some modifications. The assay was performed in a volume of 100 µl. For each sample, two tubes, the control and the sample itself were set up. A 90 µl mixture containing 65 µl cell suspension, 5 µl 20 mM thiamin diphosphate (ThDP), 5 µl 0.2 M MgCl₂, 5 µl of 4 mM FAD and 10 µl of 1 M K₃PO₄ (pH 7.5) was added to each tube. After incubation at 30 °C for 10 min, 10 µl 1 M pyruvate was added to the mixture. The reactions were stopped after 20 min at 30 °C by the addition of 11.3 µl 9.9 M H₂SO₄ to the sample and 150 µl 6 M NaOH to the control tube. The sample tubes were then incubated at 60 °C for 30 min to allow the efficient conversion of α -acetolactate to acetoin. After that, 140 µl 8 M NaOH was added to each sample tube to stop the reaction. At this point, the sample and control had the same volume (250 µl) and the same pH. The yield of α -acetolactate was determined by the amount of acetoin

produced in the decarboxylation reaction, which took place in the acidic conditions at high temperature. In the control tubes, α -acetolactate was not decarboxylated to acetoin; therefore the amount of acetoin produced in the decarboxylation reaction was calculated by subtracting the acetoin amount in the background (control tube) from the total acetoin amount (sample tube).

The concentrations of acetoin in the control and sample tubes were determined using a colorimetric method (Westerfeld, 1945). Each tube was filled with 750 µl water, 200 µl 0.5% creatine and 200 µl 5% α -naphthol freshly prepared in 2.5 M NaOH. The tubes were vortexed for 2 s and kept for 1 h at RT to allow color development. The reaction mixtures were centrifuged for 2 min for clarification. Absorbance of the supernatants at 525 nm was measured against the blank made up of 1 ml water, 200 µl 0.5% creatine and 200 µl 5% α -naphthol. *In vitro* AHAS activity was calculated as acetoin produced per mg of permeabilized cell protein per h. Protein concentration was determined using the Bradford assay (Bradford, 1976).

2.11. Analytical methods

Apparent extracts and vicinal diketones (GC-ECD) were determined according to MEBAK (Band III, 1.1.1, 1.1.4, 1996) and MEBAK (Band II, 1.2.1, 1996), respectively.

3. Results

3.1. Brewing-relevant phenotypes of three lager yeast strains differing in diacetyl production

The brewing performances of the three lager brewers' yeast strains with different diacetyl production characteristics (see Material and Methods) were investigated under industrially relevant conditions (hopped wort of 11.38°P, 12 °C fermentation temperature) in 3-l glass bioreactors. For each strain, the time courses of apparent extract were recorded. The apparent extract is measured by gravity and can be considered as a readout for the concentration of wort sugars. The declines in wort gravity were almost identical for strains HD and MD (Fig. 1A). However, strain LD fermented the sugars of the wort slower than the other two strains, which is obvious after 72 h of the main fermentation (Fig. 1A). Thus, compared to strains HD and MD, strain LD required significantly more time (20 h) in order to reach the end of the main fermentation. The reason for the longer fermentation time of strain LD becomes clear in Fig. 1B. This strain possessed a significant flocculation already before the end of fermentation, which can be seen by comparing the turbidity of the culture (reflecting non-sedimented cells) at day 7 of wort fermentation (168 h) in the *Lietz* fermentation devices. The early flocculation is the major reason why strain LD itself is not relevant for industrial beer brewing even though its diacetyl formation seems to be ideal (see below),

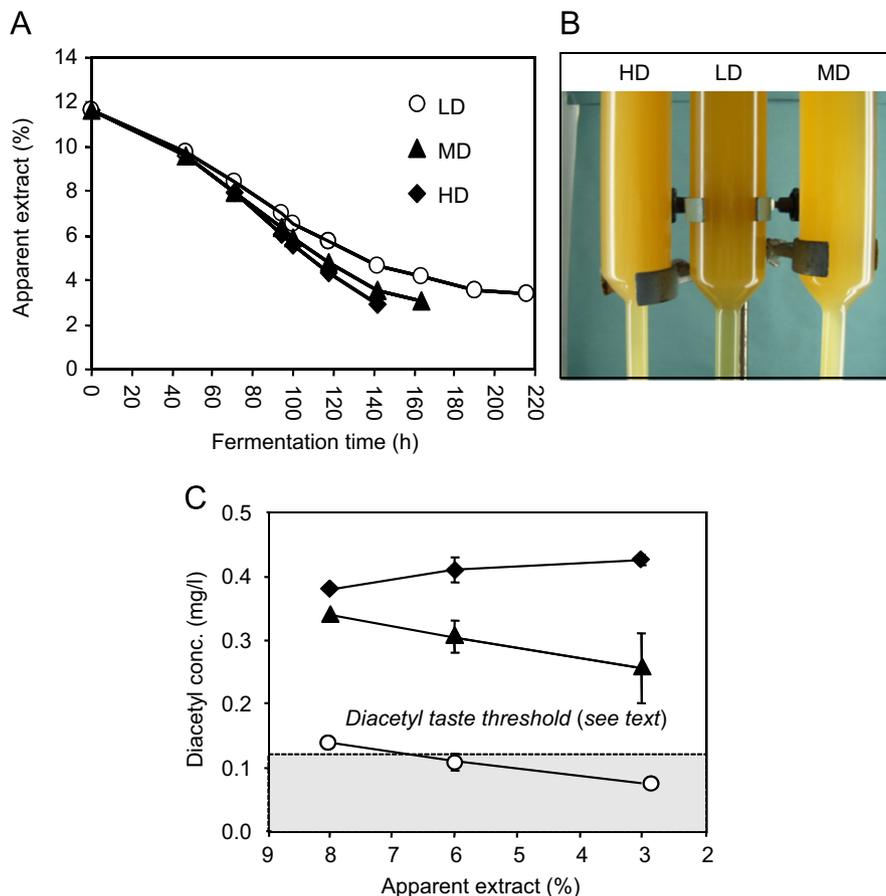


Fig. 1. Relevant phenotypic traits of three selected lager brewers' yeast strains showing marked differences in diacetyl formation. Fermentations were carried out under conditions relevant to industrial brewing (11.38°P brewers' wort, 12 °C) in 3-l glass bioreactors with slight stirring of 50 rpm (A and B) or *Lietz* devices (C). (A) Time course of apparent extract. (B) Flocculation behavior. The clearance of the culture can be used as readout for cell sedimentation. The picture was taken after 7 days (168 h) of fermentation. (C) Diacetyl production. In order to allow better strain comparison, diacetyl concentration was plotted against the apparent extract. The diacetyl levels shown for apparent extracts of 6% and 3% are mean values from two independent experiments, including standard deviations. Diacetyl levels shown for apparent extract of 8% were taken from a single experiment. Values reflect the total diacetyl concentration in the culture supernatant, i.e. the sum of free diacetyl and α -acetolactate.

For each strain, total diacetyl concentration (i.e. the sum of free diacetyl and α -acetolactate) was measured when wort reached an apparent extract of 8%, 6% and 3%. At an apparent extract of 8% cells were in the exponential growth phase while they reached their max. concentration of non-sedimented cells at an apparent extract of 6% (data not shown). An apparent extract of 3% reflects the end of the fermentation (Fig. 1A). In order to show relative differences in diacetyl production, the total diacetyl concentration was plotted against the apparent extract for each strain (Fig. 1C). Strain HD showed the highest diacetyl levels, while the diacetyl concentrations of strain MD were moderate. The Fig. 1C also shows that the diacetyl levels of strain LD were considerably lower compared to strains HD and MD at all time points. For example, the measured diacetyl concentration for strain LD at the end of the fermentation was only 17% and 34% compared to MD and HD, respectively. Diacetyl concentration of strain LD was even in a range below the taste-threshold for diacetyl in lager beer, which is between 0.017 and 0.15 mg/l (Saison et al., 2009).

3.2. Comparative analysis of the three lager brewers' yeast strains at the level of transcriptome and genome

We carried out microarray-based transcriptome analyses and comparative genome hybridizations. The cell samples used for transcriptome studies were harvested at an apparent extract of 8% of the main fermentation where all three strains were in the

exponential growth phase and showed comparable cell densities (data not shown). The microarray-based analyses were carried out using customized lager brewers' yeast microarrays (see Material and Methods) based on the recently published genomic sequence of the commonly used lager brewers' yeast strain Weihenstephan 34/70 (Nakao et al., 2009). As these arrays contained probes for both Sc- and Sb-type genes of lager brewers' yeast, they enabled a reliable in-depth analysis. This is in notable contrast to many previous microarray-based global studies on lager brewers' yeast, which relied on commercially available microarrays where probe sequences were derived from the published genomic sequence of the laboratory *S. cerevisiae* strain S288c. Those studies did not allow differentiating between the two orthologs of most genes present in lager brewers' yeast.

The brewers' yeast specific microarrays were used in order to study the mRNA abundance and copy numbers of the three strains at a global level. The number of transcripts that showed significant differences in the comparison of the two closely related strains (HD vs. MD) was approximately 300. In contrast, the comparison of strain LD to strain HD or MD revealed more than 1000 significant differences each. It has to be emphasized that genes were only considered to be differentially expressed if false discovery rate of the pairwise comparisons was equal or lower than 0.001 (see Material and Methods). This rather stringent criterion was used due to the fact that we used technical instead of biological replicates. Differences in mRNA abundance between strains can be caused by differences in copy number,

microarray analyses of the three studied strains. Significant differences between the strains at mRNA level were found for the following genes: *Sc-ILV6*, *Sc-BAT1*, *Sb-BAT1* and *Sb-BAT2*. Out of the four differences identified at mRNA level, three (see results for *Sc-ILV6*, *Sc-BAT1* and *Sb-BAT1*) seemed to correlate with the differences in copy-number obtained by microarray-based CGH (Fig. 2, left side). These three differences were considered as being most reliable. In contrast to the situation for *Sc-ILV6*, the expression levels/copy numbers of *Sc-BAT1* and *Sb-BAT1* did not correlate with the diacetyl production of the three strains. This evaluation of the data revealed *Sc-ILV6* expression as a probable reason for differences in diacetyl production and it seemed to be worth to study the impact of abolishing *Sc-ILV6* expression on diacetyl formation by targeted genetic engineering.

3.3. Disruption of two copies of *Sc-ILV6*: impact on diacetyl production, wort fermentation performance and beer taste

We first deleted one copy of *Sc-ILV6* in the medium diacetyl producer MD, which is a commercially used lager brewers' yeast strain. The deletion was based on homologous recombination using a disruption cassette, which contained the *loxP-KanMX-loxP* module (Gueldener et al., 2002) as shown in Fig. 3. The primer sequence used for homologous recombination was chosen to have the lowest possible homology to *Sb-ILV6* in order to avoid integration of the deletion cassette at the ortholog of *Sc-ILV6* in the hybrid yeast. After selecting the correct single deletion strain by diagnostic PCR (see Section 2), the second copy of this gene was deleted by applying the same principle and primers as for the deletion of the first *Sc-ILV6* copy (Fig. 3) but using the *loxP-ble^r-loxP* module (Gueldener et al., 2002) as the selectable marker. As diagnostic PCR of the *Sc-ILV6* double deletion did not reveal any additional copy of this gene, we concluded that strain MD was diploid for this sequence. By choosing sequence specific primers for both *Sc-ILV6* and *Sb-ILV6*, we were able to distinguish between both orthologs (see Section 2) and made sure that solely the two copies of *Sc-ILV6* were deleted while *Sb-ILV6* copies remained unchanged.

ILV6 is proposed to encode a regulatory subunit of aceto-hydroxyacid synthase (*Ilv2p*), which catalyzes the conversion of pyruvate to α -acetylactate (Fig. 2; see Section 4). It could well be

that differences in AHAS activity are responsible for the variations in α -acetylactate and diacetyl formation in the studied strains. In fact, a reduced *Ilv2p* protein level has been shown to result in reduced diacetyl production (Liu et al., 2004). Therefore, we measured *in vitro* AHAS activity in the *Sc-ILV6* single and double deletion mutants as well as in the non-modified strain MD (100 ml-scale brewers' wort fermentations). As shown in Fig. 4, the reduction of *in vitro* AHAS activities caused by deletion of one and two copies of *Sc-ILV6* was not significant. For comparison, we also measured the *in vitro* AHAS in strain LD (i.e. the natural low-diacetyl producer) but it was also insignificantly lower compared to the unmodified strain MD (Fig. 4). However, when measuring diacetyl production in the *Sc-ilv6* single and double deletion strains, the reduction in diacetyl concentration compared to the unmodified strain was about 13% and 40% by the single and double deletion, respectively. While the diacetyl reduction in the single deletion strain was insignificant (*p*-value above 0.05), the reduction in the double deletion strain was significant according to student *t*-test (*p*-value: 0.016).

The diacetyl formation of the *Sc-ilv6* double deletion strain and the unmodified reference strain MD was also studied in larger scale, i.e. in a 25-l brewers' wort fermentation. This allowed more samplings and the recording of diacetyl time courses. When comparing the time course of diacetyl production for the reference strain MD in Fig. 5A with the one in Fig. 1, it has to be considered that different batches of brewers' wort were used, which might have caused slight differences. Brewers' wort is a non-defined medium and composition can slightly change from batch to batch. This can result in differences in the diacetyl time courses since diacetyl formation and its regulation is highly complex and particularly dependent on FAN content of the wort (see introduction). However, the diacetyl production of the *Sc-ilv6 Δ* double deletion strain was always remarkably lower compared to the reference strain at all apparent extracts (Fig. 5A). This result could be confirmed in another batch of wort even though the absolute values slightly differed between wort batches (data not shown). The diacetyl level of the *Sc-ilv6 Δ* double deletion strain at the end of the fermentation (i.e. at an apparent extract of 2.8%) was only 35% in comparison to the wild type. The actual concentration was even in the diacetyl taste threshold range in beer (see above). The curves for 2,3-pentanedione production strongly resemble those of strains' diacetyl production

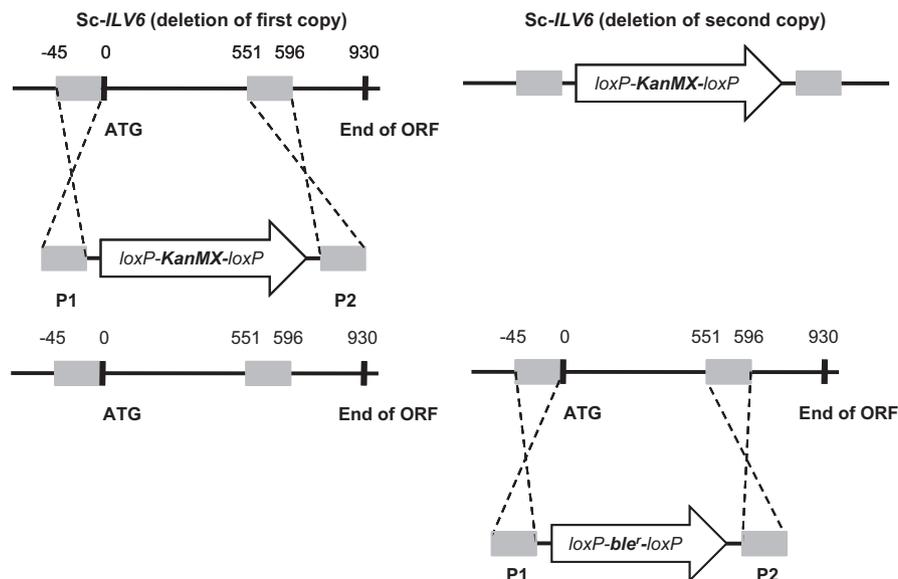


Fig. 3. Strategy employed for deletion of the two copies of *Sc-ILV6* in a lager brewers' yeast strain.

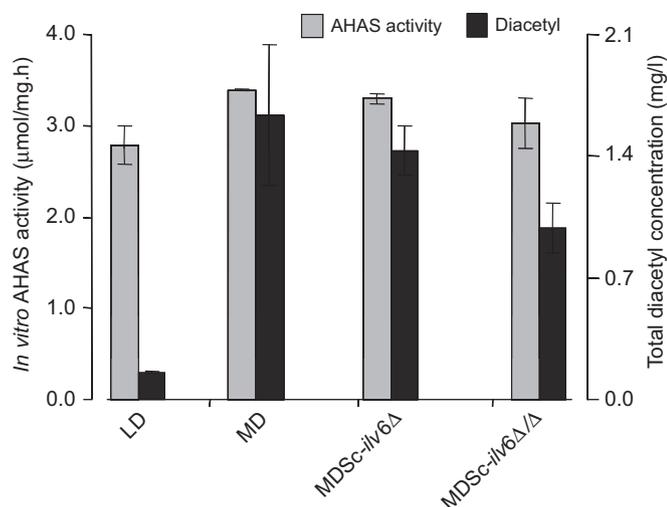


Fig. 4. *In vitro* activity of acetohydroxyacid synthase (AHAS, Ilv2p) and diacetyl production in a commercially used lager brewers' yeast strain (MD) after deletion of one (*Sc-ilv6Δ*) and two copies of *Sc-ILV6* (*Sc-ilv6Δ/Δ*). For comparison, strain (LD) was also included in this experiment. Both protein extracts for AHAS enzyme activity measurements and culture supernatants for diacetyl determination were taken from brewers' wort fermentations at an apparent extract of roughly 8.5% (varying from 8.3 to 8.8% for the different strains). Diacetyl values reflect the total diacetyl concentration in the culture supernatant, i.e. the sum of free diacetyl and α -acetolactate. Data shown are mean values of three independent experiments including standard deviations.

(Fig. 5A and B). This compound was measured since it is also produced by the activity of AHAS in a parallel pathway. The growth of the *Sc-ilv6Δ/Δ* double deletion strain during the main fermentation was similar to that of the reference strain MD (data not shown). Nevertheless, there was a slight reduction in wort sugar consumption for the *Sc-ilv6Δ/Δ* double deletion strain in the second phase of the main fermentation (Fig. 5C). Thus, *Sc-ilv6Δ/Δ* double deletion strain needed 8 days to reach wort attenuation compared to 7 days required for the reference strain. The green beer was subjected to maturation (second fermentation) and afterwards tasted by a panel of experts. The beer brewed with the *ilv6Δ/Δ* double deletion strain did not show any significant changes in beer flavor or taste (data not shown).

4. Discussion

Diacetyl reduction is one of the major demands in lager brewers' yeast optimization since this could remarkably reduce the time required for second fermentation (Helbert, 1982). Using diacetyl reduction as an exemplified target, we present the proof-of-concept of exploiting natural diversity of lager brewers' yeast strains for strain improvement. Three lager brewers' yeast strains with different diacetyl production levels were subjected to global molecular analysis at two different molecular levels. Due to the huge number of significant differences between the three strains obtained after microarray-based global comparisons, we first concentrated global data evaluation to the six genes encoding the proteins involved in the valine biosynthetic pathway. In fact, it was most likely that the three strains show differences in this well known pathway. Among the six genes considered here, *Sc-ILV6* was the only gene for which copy number and mRNA abundance in the three different strains matched well with the corresponding diacetyl formation. Based on this result, we deleted the two copies of *Sc-ILV6* present in strain MD and successfully reduced the diacetyl production in this commercially used lager yeast strain. It has to be emphasized that the ortholog (*Sb-ILV6*) did not show any significant strain-to-strain differences

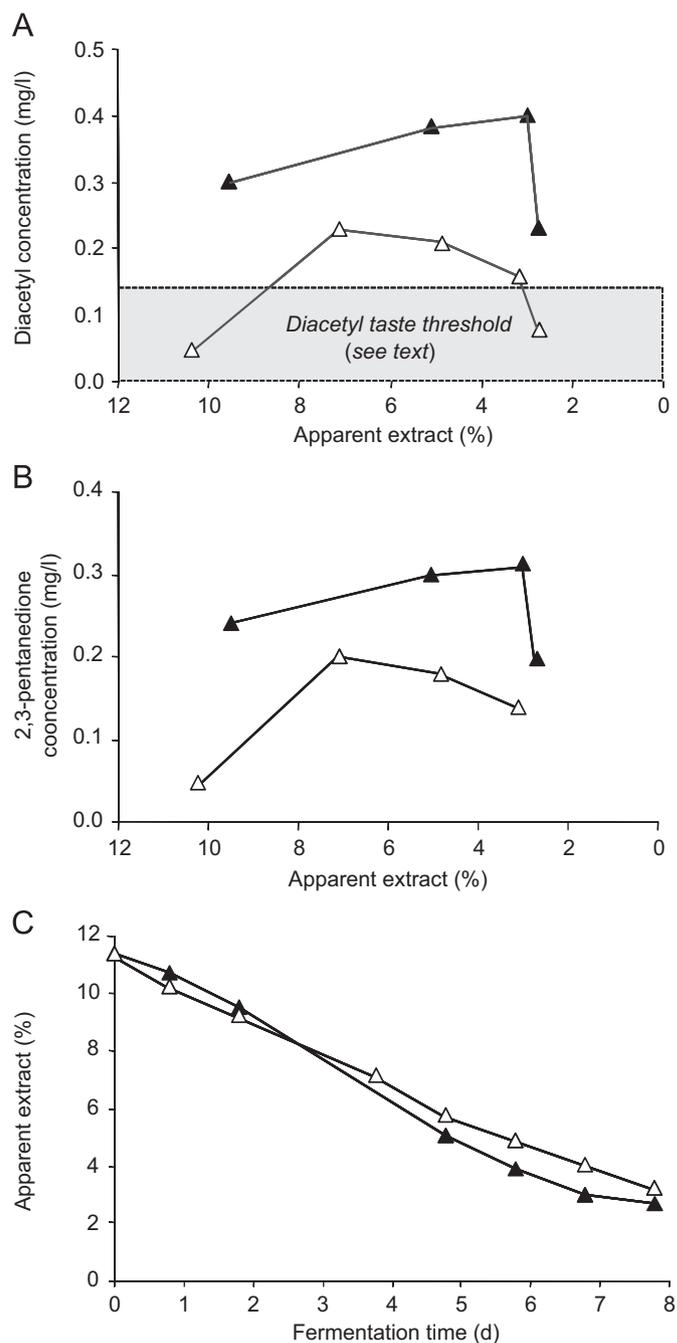


Fig. 5. Time-courses of diacetyl (A), 2,3-pentanedione (B) and wort sugar consumption (C) of a commercially used lager brewers' yeast strain (MD) deleted in two copies of *Sc-ILV6* (Δ) compared to the unmodified reference strain MD (\blacktriangle). Fermentations were carried out in 25-l scale at 10.5 °C using brewers' wort (11.38°P). The data from one representative experiment are shown. In total, three independent experiments using two different batches of brewers' wort were carried out.

(Fig. 2). This result suggests that the low diacetyl phenotype is caused by different expression of the *Sc*-allele but not the *Sb*-allele. Thus, we made sure that the *Sb-ILV6* remained untouched during the construction of the *Sc-ILV6* gene double deletion strain.

ILV6 is assumed to encode a subunit of AHAS (Ilv2p). Most research on AHAS has been conducted in enterobacteria where three active AHAS isoenzymes (AHASI, AHASII and AHASIII) exist (McCourt and Duggleby, 2006). All three isoenzymes have a tetrameric structure ($\alpha 2\beta 2$) consisting of two large and two small subunits. Catalytic activity is conferred by the large subunits while the small subunits may affect feedback regulation, enzymatic activity, and

stability. In *S. cerevisiae*, only one isoenzyme of AHAS has been found. The catalytic subunit is nuclear-encoded by *ILV2* and targeted into mitochondria. When the whole genome sequence of *S. cerevisiae* was available, homology search revealed that the protein encoded by *YCL009c* (*ILV6*) is homologous to the small subunits of prokaryotic AHAS. The disruption of *ILV6* in a laboratory strain of *S. cerevisiae* did not result in a reduction of AHAS activity measured in permeabilized cells. However, AHAS activity of the *ilv6* deletion mutant was resistant to inhibition by valine (Cullin et al., 1996). Based on this result, it has been assumed that *ILV6* mediates *in vivo* feedback regulation of AHAS by valine. Pang and Duggleby (1999) separately cloned and expressed both *ILV2* and *ILV6* in *E. coli* and afterwards reconstituting both subunits. A substantial (7-fold) enhancement of the AHAS enzyme activity was observed after reconstitution under optimal conditions. This result supports the assumption of *Ilv6p* being an enhancer of *Ilv2p* but is in contradiction to the above mentioned finding of Cullin et al. (1996) showing that *ILV6* deletion did not result in any reduction of AHAS activity *in vitro*. Our own results shown here are puzzling as well. Although the *in vitro* AHAS activity measured in permeabilized cells was not significantly reduced in the *Sc-ILV6* double deletion strain, there was a clear *in vivo* impact of *Sc-ILV6* disruption on diacetyl production.

According to our results, low *Sc-ILV6* expression cannot be the only rationale for low diacetyl production of strain LD at molecular level. In fact, the diacetyl production of strain LD was significantly lower compared to the double deletion strain *Sc-ilv6Δ/Δ* (Fig. 4). We so far checked the single-copy overexpression of *Sb-BAT1* in the *Sc-ILV6* double deletion strain based on our results shown in Fig. 2. Indeed, we saw a slight reduction in *Sc-ilv6Δ/Δ+Sb-BAT1* compared to the double deletion strain bearing the empty plasmid (data not shown). The pending analysis of the remaining genes could result in the identification of further so far unknown targets for diacetyl reduction. However, it has to be realized that the identification and verification of potential targets out of microarray data has been challenging. Even after integrating our two data sets, the number of significant differences was too high in order to verify all of them. In fact, the only way for verification is genetic engineering (i.e. target gene overexpression or reduction/deletion of gene expression). As already mentioned, lager brewers' yeast strains contain homologous *Sb*-type orthologs in addition to the *Sc*-type genes, which strongly compromise allele-specific gene deletions. In addition, industrial strains are less accessible to transformation and genomic integration by homologous recombination as also discussed by Blicek et al. (2007). Our study underlines the urge to develop better tools for evaluation and integration of global molecular analysis data for brewers' yeast.

Nevertheless, our *Sc-ilv6* double deletion mutant (or a corresponding self-cloned strain without any heterologous DNA sequences) resulting in 65% reduction of final diacetyl concentration should be a very desirable strain for breweries. Except a slight increase in fermentation time (7 instead of 6 days), the genetic modification did not negatively influence the brewing characteristics of the yeast and the taste of the beer.

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Appendix A. Supplementary Materials

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ymben.2011.07.005.

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