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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.

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 Kurztman, 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; Villanueba 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 DH5α™ (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 (OD_{600}). 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 Sc*ilv6*Δ	Sc-*ilv6*Δ	Sc-*ILV6*/Sc-*ilv6*Δ:: *loxP-KanMX-loxP*	This study
Sa-06165 Sc-*ilv6*Δ/Sc*ilv6*Δ	Sc-*ilv6*Δ/Δ	Sc-*ilv6*Δ::*loxP-KanMX-loxP*/Sc-*ilv6*Δ:: *loxP- ble*^{r}*–loxP*	This study