

# Reducing levels of diacetyl in saccharomyces pastorianus



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## A critique of approaches to understand and reduce levels of diacetyl in *saccharomyces pastorianus*

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### Section 1: Background and Context

Diacetyl is a vicinal diketone (VDK), produced as a by-product of intermediates in the valine biosynthetic pathway of yeast lager stains (Krogerus and Gibson, 2013b). It causes an unfavourable butterscotch flavour (Gibson et al., 2014). Yeast can remove diacetyl, but the process requires a long maturation fermentation, costing time, efficiency and energy (Krogerus and Gibson, 2013b). If diacetyl levels can be reduced to under taste threshold (20-100 ppb) (Krogerus and Gibson, 2013a) faster, without affect beer quality, then the maturation time can be shortened (Kusunoki and Ogata, 2012). The following studies have modified existing yeast strains to understand and reduce diacetyl levels. The use of genetically modified organisms is avoided due to a lack of consumer support for this approach (Kusunoki and Ogata, 2012).

### Section 2: Description of experimental work and finding in this area

Duong et al. examined global genomic analysis of three yeast strains, HD (High Diacetyl), MD (Medium Diacetyl) and LD (Low Diacetyl) with different diacetyl production levels. They restricted the results to genes involved in the valine biosynthetic pathway (Duong et al., 2011). Results showed a low expression in *Sc-ILV6*, indicating it as a target to reduce diacetyl levels (Duong et al., 2011). mRNA abundance and gene copy number showed a

correlation between Sc- *ILV6*, Sc- *BAT1* and Sb- *BAT1* (Duong et al., 2011). The study choose to monitor Sc- *ILV6*, as the others did not correlate with diacetyl production – the reason for this remains unclear. One copy of Sc- *ILV6* was deleted in strain MD, using the “*loxP-KanMX-loxP*” disruption cassette (Duong et al., 2011). Diagnostic PCR identified the correct strain and the second copy of the gene was deleted using the “*loxP-ble<sup>r</sup>-loxP*” disruption cassette to allow selection of the transformant (Duong et al., 2011). A strength of the study was that it avoided mutation in the Sb- *ILV6* copies, preventing a 3<sup>rd</sup> variable affecting the results. It was surprising to note that the single and double deletion of Sc- *ILV6* had no major effect on acetohydroxyacid synthase (AHAS) activity (Duong et al., 2011) (*ILV6* produces a regulatory subunit of this enzyme), despite these strains being able to reduce diacetyl levels by 13% and 40% respectively (Duong et al., 2011). The reason for this was not addressed. The deletion did not affect beer quality (Duong et al., 2011). Strain LD was not used as it was a slow fermenter with early flocculation (Duong et al., 2011). Different batches of wort were utilised all through the study (Duong et al., 2011) and breweries may be cautious as this industrial condition was not kept constant.

Kusunoki and Ogata inserted the *ILV5* gene into *Saccharomyces pastorianus* (FY-2) with low diacetyl production to increase the metabolism of  $\alpha$ -acetolactate – a diacetyl precursor (Kusunoki and Ogata, 2012). Fusion PCR created a 5.3 kb cassette containing SC- *ILV5* and *SMR1B*, a sulfometuron methyl (SM) resistance gene (Kusunoki and Ogata, 2012). This was inserted into the DNA, upstream of SC- *ILV2* for transformation (Kusunoki and Ogata, 2012). SC- *ILV2* was substituted for *SMR1B* and resistant transformants were

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cultured (Kusunoki and Ogata, 2012). PCR confirmed the cassette was inserted into the correct site. One transformant amplified a 5.7 kb product (Kusunoki and Ogata, 2012), demonstrating the construct was introduced upstream of both alleles of SC- *ILV2* (FY-2/SC- *ILV5* homo) and another transformant formed a 2.9 kb and a 5.7 kb fragment (Kusunoki and Ogata, 2012) verifying insertion upstream of one allele of SC- *ILV2* (FY-2/SC- *ILV5* hetero). Another PCR and Sequencing verified the presence of the construct. Sequencing also confirmed that in the FY-2/SC- *ILV5* homo, SC- *ILV2* was substituted by *SMR1B* in one allele only (Kusunoki and Ogata, 2012). Fermentation with FY-2/SC- *ILV5* homo took 67 hours to complete and resulted with a VDK level below threshold values (Kusunoki and Ogata, 2012). FY-2/SC- *ILV5* hetero took 160 hours to complete fermentation and did not have a major effect on VDK levels compared to the parent strain (Kusunoki and Ogata, 2012). Another fermentation was performed to assess flavour components (Kusunoki and Ogata, 2012). Headspace solid-phase microextraction gas chromatography and Mass spectrometry all confirmed flavour compounds were unaffected (Kusunoki and Ogata, 2012). A limitation of the study was the failure to compare the levels of flavour components were similar in both fermentations, to eliminate any 3<sup>rd</sup> variable affecting the results.

Valine provides feedback inhibition of enzymes catalysing the production of diacetyl precursors. 3 fermentations were performed with *S. pastorianus* (Krogerus and Gibson, 2013b). High-performance anion exchange chromatography (HPAEC) analysed fermentable sugars (Krogerus and Gibson, 2013b). Headspace gas chromatography and Mass spectrometry

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analysed total VDK and aroma compounds. Chromatography analysed amino acid content (Krogerus and Gibson, 2013b). The first fermentation added increasing concentrations of valine to the wort, the highest addition (300mg/L) lowering diacetyl concentrations by 37% during fermentation and by 33% at the end of fermentation (Krogerus and Gibson, 2013b). Diacetyl levels were not reduced below threshold (Krogerus and Gibson, 2013b). The second fermentation investigated adding valine to worts with standard and reduced FAN levels, resulted in reduced FAN having the lowest diacetyl levels during and at the end of fermentation (Krogerus and Gibson, 2013b). Diacetyl concentrations were not reduced below threshold (Krogerus and Gibson, 2013b). Valine-supplemented standard FAN demonstrated a negative correlation between the valine uptake rate and the diacetyl level during fermentation (Krogerus and Gibson, 2013b). The study neglected to mention this effect with respect to reduced FAN wort. The third fermentation investigated the effect of doubling the wort content of amino acids groups on levels of diacetyl and the difference in valine uptake (Krogerus and Gibson, 2013b). Branched-Chain Amino Acids (BCAA) showed the lowest diacetyl levels (Krogerus and Gibson, 2013b). No supplement decreased diacetyl levels below threshold. Non Preferred Amino Acid (NPAA) supplemented wort demonstrated a negative correlation between the uptake of valine and diacetyl level (Krogerus and Gibson, 2013b). Altering amino acid spectrum has an indirect effect on diacetyl levels (Krogerus and Gibson, 2013b). A strength of the study is that they ensured the yeast was conditioned to be similar to yeast used in industrial fermentations, making the results industrially relevant.

Two *S. pastorianus* strains, A153 with low diacetyl formation and W34, with higher diacetyl formation was selected by fermentation (Gibson et al., 2014). The strains showed similarities in fermentation parameters such as fermentation rate, aroma compounds and biomass growth (Gibson et al., 2014).

A second fermentation analysed the gene transcription of orthologous *ILV* genes in A153 and W34, to examine if differences in diacetyl formation between the strains correlated to differences in expression of genes involved in valine biosynthesis (Gibson et al., 2014). This fermentation revealed Sc *ILV6* corresponded with diacetyl formation (Gibson et al., 2014) as previously shown by (Duong et al., 2011). The *ILV6* gene, which produces a regulatory subunit of AHAS, showed transcription after 6 hours with 5 times more expression in W34 than A153 (Gibson et al., 2014). Both *S. cerevisiae* and *S. eubayanus* copies of *ILV6* showed this early transcription (Gibson et al., 2014), although Sc *ILV6* in W34 demonstrated higher transcription than Sc *ILV6* in A153 (Gibson et al., 2014).

A third fermentation investigated the function of *ILV6* in regulating diacetyl formation and analysis of the functional difference between the two forms of the gene (Gibson et al., 2014). A153 and W34 were modified to overexpress one form of *ILV6* or the other and these strains were used to ferment wort in standard conditions (Gibson et al., 2014). Overexpression of either form of *ILV6* revealed two times more diacetyl amount relative to the control (Gibson et al., 2014), in both overexpressed strains, indicating a lack of functional divergence and verifying the role of *ILV6* in regulating diacetyl formation (Gibson et al., 2014). Both strains demonstrated continued transcription of <https://assignbuster.com/reducing-levels-of-diacetyl-in-saccharomyces-pastorianus/>

Sc *ILV2*, which encodes AHAS (Gibson et al., 2014). Transcription results are consistent with native type A153 and W34 (Gibson et al., 2014).

Headspace GC-MS examined levels of alcohols, aroma compounds and total diacetyl. High performance liquid chromatography analysed residual sugars. Fermentation performance was unaffected (Gibson et al., 2014).

### Section 3: Analysis of the current state of knowledge

MDS- *ilv6*  $\Delta/\Delta$  strain showed diacetyl levels decreased below taste threshold at the end of fermentation (Duong et al., 2011). However, this strain took 8 days to complete fermentation, compared to 7 days in the control strain (Duong et al., 2011). The study noted a lack of strain-to-strain differences between Sc- *ILV6* and Sb- *ILV6*, despite a higher diacetyl concentration for Sb- *ILV6*, suggesting different regulation of gene expression of the two genes (Duong et al., 2011).

The use of FY-2/SC- *ILV5* -homo may be highly significant in industry, as it takes less time to produce levels of diacetyl under threshold values, without affecting beer quality (Kusunoki and Ogata, 2012).

Valine addition to brewer's wort lowers diacetyl throughout fermentation (Krogerus and Gibson, 2013b). Results show higher valine uptake rate into yeast (Krogerus and Gibson, 2013b), therefore valine's regulation of AHAS causes low diacetyl concentrations (Krogerus and Gibson, 2013b).

Decreasing FAN content lessened the level of diacetyl (Krogerus and Gibson, 2013b), but another study found if FAN was reduced again, diacetyl levels increased (Pugh T, 1997).

Results prove *Ilv6* protein regulates diacetyl formation in fermentation (Gibson et al., 2014). *Ilv6* subunit controls AHAS activity to produce diacetyl precursors (Gibson et al., 2014). There is no functional difference between the two types of *Ilv6* (Gibson et al., 2014).

#### Section 4: General Significance and future directions

When the diacetyl level of MDSc- *ilv6*  $\Delta/\Delta$  strain is compared with the LD strain, LD has a lower diacetyl level. This indicates that low expression of *ILV6* is not the only factor affecting diacetyl level (Duong et al., 2011), there may be other genes that are targets to obtain low diacetyl levels.

The yeast strain used is crucial, as overexpression of *ILV5* can affect flavour compounds (Kusunoki and Ogata, 2012). Both (Omura, 2008) and (Villanueva et al., 1990) reported increased flavour components, but Villanueva's beer quality was unaffected (Villanueva et al., 1990). (Kusunoki and Ogata, 2012) also used a derivative of a commercially used yeast strain like Omura, but Omura's strain produce higher VDK levels (Omura, 2008). The reason for the differences in VDK levels between the two transformants in this study is unknown and it is interesting to note that another study reported a similar result (Iijima and Ogata, 2010). The study assumes homo-insertion correlates to higher gene expression compared to hetero-insertion (Kusunoki and Ogata, 2012).

Valine addition shortens maturation time (Krogerus and Gibson, 2013b), which is significant to breweries. Fermentation performance, yeast biomass and pH remained unchanged by supplements (Krogerus and Gibson, 2013b).

Amino acid supplementation caused a minor effect on flavour compounds  
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(Krogerus and Gibson, 2013b), so it is essential to regulate the amount amino acids as high levels of amino acids could affect beer quality.

An investigation needs to be reported on why *Sc ILV6* has a greater effect on diacetyl precursor formation than *Se ILV6* (Gibson et al., 2014). This could be done by analysing the copy numbers of the orthologous genes and noting differences in transcription (Gibson et al., 2014). We remain unclear as to how the *Ilv6* p subunit regulates AHAS (Gibson et al., 2014).

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