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125th Anniversary Review: Diacetyl and its control during brewery fermentation

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Diacetyl is a butter-tasting vicinal diketone produced as a by-product of yeast valine metabolism during fermentation. Concentration is dependent on a number of factors including rate of formation of the precursor α-acetolactate by yeast, spontaneous decarboxylation of this acetohydroxy acid to diacetyl and removal of diacetyl by yeast via the action of various reductase enzymes. Lowering concentrations of diacetyl in green beer represents an expensive and time-consuming part of the brewing process and strategies to minimize diacetyl formation or hasten its reduction have potential for improving overall efficiency of the lager brewing system. Here we review the processes that determine diacetyl levels in green beer as well as the various ways in which diacetyl levels can be controlled. The amount of diacetyl produced during fermentation can be affected by modifying process conditions, wort composition or fermentation technique, or by yeast strain development through genetic engineering or adaptive evolution. The process of diacetyl reduction by yeast is not as well understood as the process of formation, but is dependent on factors such as physiological condition, cell membrane composition, temperature and pH. The process of diacetyl removal is typically rate-limited by the reaction rate for the spontaneous decarboxylation of α-acetolactate to diacetyl.

Keywords: diacetyl; vicinal diketone; amino acid; beer; yeast; fermentation

Diacetyl production during fermentation

Diacetyl (2,3-butanedione) and 2,3-pentanedione are vicinal diketones (VDK) formed during beer fermentation as by-products of amino acid synthesis (valine and isoleucine, respectively) in *Saccharomyces* yeast, and these can have a significant effect on the flavour and aroma of beer. Diacetyl is known for its butter- or butterscotch-like flavour, with a flavour threshold usually reported as around 0.1–0.2 ppm in lager and 0.1–0.4 ppm in ales *(1,2)*, although flavour thresholds as low as 17 ppb *(3)* and 14–61 ppb *(4)* have been reported. It has also been suggested that the flavour threshold of diacetyl varies with taster's geographical background, ethnicity and diet *(5)*. The compound 2,3-pentanedione has a similar flavour to diacetyl, although often described as more toffee-like, but with a higher flavour threshold of around 0.9–1.0 ppm *(1,2)*. VDKs are most easily detectable in lighter beers, where the flavour is not masked by malt and hop flavours. The presence of VDKs above their flavour threshold in beer is generally regarded as a defect, since their flavour is undesirable in many beer styles and it can also indicate microbial contamination, for example, by *Lactobacillus* spp., *Pediococcus* spp. or *Pantoea agglomerans (6–8)*. Nevertheless, diacetyl at detectable concentrations is acceptable in some beer styles, such as Bohemian Pilsner and some English ales. Martineau *et al. (9)* found diacetyl concentrations ranging from 0.002 to 0.1 ppm (average concentration of 0.046 ppm) during the analysis of 11 commercial American beers. Diacetyl concentrations in beer can be determined via a variety of analytical methods, including colorimetric assays (e.g. through complex formation with dimethylglyoxime or *o*-phenylenediamine), gas chromatography and liquid chromatography *(10–12)*. During analysis, care must be taken in order to avoid interference by 2,3-pentanedione and α-acetolactate.

The generally accepted pathways for diacetyl and 2,3-pentanedione formation and reduction in *Saccharomyces* spp. are presented in Fig. 1 *(13–16)*. Diacetyl and 2,3-pentandione are formed indirectly as a result of valine and isoleucine anabolism, since they arise from the spontaneous non-enzymatic oxidative decarboxylation of α-acetohydroxy acids that are intermediates in the valine and isoleucine biosynthesis pathways. Valine and isoleucine synthesis is localized in the mitochondria *(17)*. In the former pathway, the reaction between α-acetolactate and 2,3-dihydro-isovalerate is rate-limiting, and thus during fermentation and yeast growth, α-acetolactate is secreted out through the cell membrane into the wort *(13,16–19)*. The reasons and mechanisms for α-acetolactate secretion are not fully understood, but may involve protecting the yeast from carbonyl

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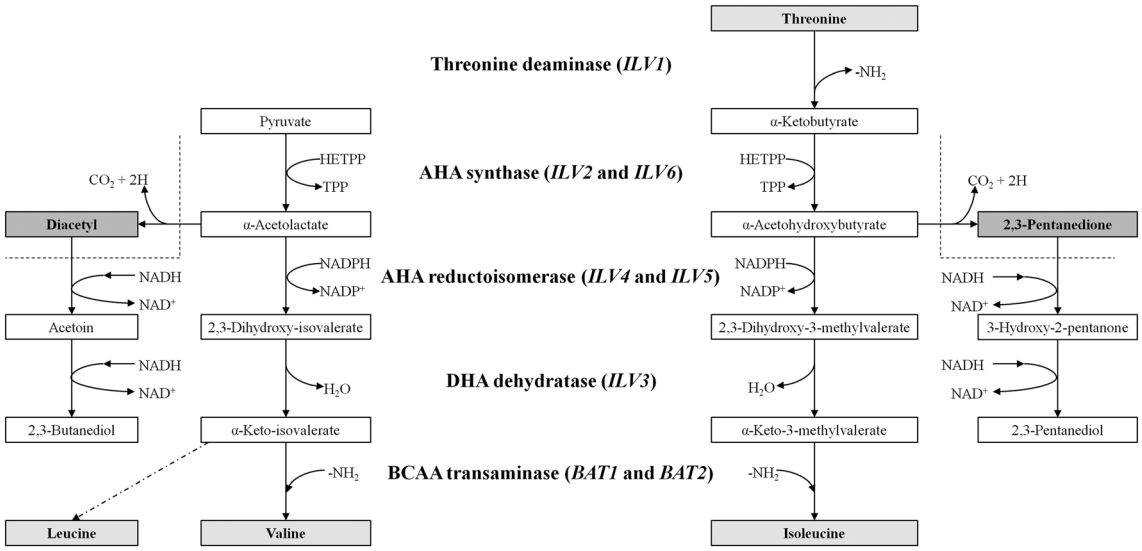

Figure 1. The pathways for diacetyl and 2,3-pentandione formation and reduction, as well as valine and isoleucine synthesis, in *Saccharomyces* spp. yeast. AHA, acetohydroxy acid; DHA, dihydroxyacid; BCAA, branched chain amino acid *(13–16)*.

stress *(20)*. The α-acetolactate then spontaneously decarboxylates, either oxidatively or non-oxidatively, forming either diacetyl or acetoin respectively, and in both cases releasing carbon dioxide. The non-oxidative decarboxylation into acetoin can be encouraged by heating under anaerobic conditions and by maintaining a low redox potential in the wort *(21)*. Diacetyl production thus increases with increasing valine biosynthesis, which in turn depends on the cell's need for and access to valine and other amino acids. Hence, fermentation conditions favouring rapid yeast growth can give rise to increased diacetyl production if wort free amino nitrogen content is insufficient. A plot showing the concentrations of diacetyl and α-acetolactate during a lager beer fermentation is presented in Fig. 2. During fermentation, the concentrations of free diacetyl in wort are usually low and α-acetolactate rather constitutes the majority of the 'total diacetyl' present *(22–24)*. As a result, diacetyl concentrations are often expressed as 'total diacetyl' concentrations, that is, the sum of the free diacetyl and α-acetolactate ('potential diacetyl'), during analysis, in order to highlight potential diacetyl concentrations.

Valine uptake

The assimilation and metabolism of wort nitrogen compounds play an important role in fermentation performance, as during yeast growth cells require nitrogen to synthesize new enzymatic and structural proteins. The nitrogen sources available for yeast metabolism in wort are mainly amino acids, ammonium and shorter peptides. In *Saccharomyces* spp., these compounds are rarely used directly as building blocks for the biosynthesis of macromolecules, for example, yeast proteins, rather, they are catabolized, and any required amino acids are synthesized from catabolic intermediates *(25)*. A minimum free amino nitrogen (FAN) content in wort of 100 ppm is usually recommended for maintaining healthy yeast and a sufficient fermentation rate, although successful fermentations of wort with a FAN content of 51 ppm have been performed *(25–27)*. FAN contents above 150 ppm are mentioned by various sources for optimum fermentation performance *(27,28)*. Insufficient FAN content in wort may lead to slow fermentation rates and incomplete fermentation (i.e. low attenuation). In environments with excess FAN, the fermentation rate and ethanol productivity are increased, since carbon flow through the glycolytic pathway is maximized (as no glycolytic products need to be utilized as carbon skeletons in amino acid synthesis), but the formation of higher alcohols (such as iso-butanol) may also be increased, as many are formed from intermediates in amino acid catabolic pathways, for example, iso-butanol is formed from α-ketoisovalerate, an intermediate in the valine anabolic and catabolic pathways *(27,29)*. Yeast growth rate is also increased with increased concentrations of FAN in the wort *(30)*.

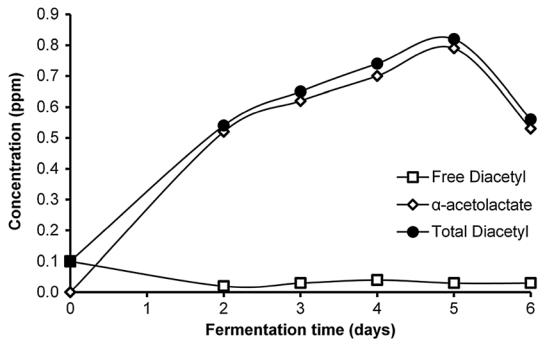

Figure 2. Concentrations of diacetyl and α-acetolactate during a lager beer fermentation. Values are derived from Haukeli and Lie *(22)*.

The amino acids present in wort have been divided into four groups by Jones and Pierce *(31)*, depending on their general uptake rate in yeast. This classification was originally based on a limited number of yeast strains and worts and does not necessarily apply to all different strains of brewing yeast. These four amino acid groups are presented in Table 1 *(31)*. The Group A amino acids were characterized by fast absorption into the yeast cells (usually complete absorption within 20 h); Group B amino acids were more gradually absorbed into the yeast cell; Group C amino acids were slowly absorbed into the cell after a 'lag phase' of around 12 h, while proline, despite being the most abundant amino acid in wort, is only marginally absorbed. Other studies *(32–34)* on the absorption of amino acids by brewing yeast in brewery conditions report similar classifications, with

Table 1. The classification of wort amino acids based on their uptake rate in *Saccharomyces cerevisiae (31)*

Group A	Group B	Group C	Group D
Fast absorption	Moderate absorption	Slow Absorption	No absorption
Glutamic acid	Valine	Glycine	Proline
Aspartic acid	Methionine	Phenylalanine	
Asparagine	Leucine	Tyrosine	
Glutamine	Isoleucine	Tryptophan	
Serine	Histidine	Alanine	
Threonine		Ammonia	
Lysine			
Arginine			

minor regrouping of some amino acids (such as reclassifying methionine as a Group A amino acid).

The uptake rate of valine is of significance when investigating diacetyl production during beer fermentation because of the link between valine synthesis and diacetyl formation. Valine has a moderate uptake rate in yeast, and as shown, for example, by Garcia *et al*. *(35)* and Perpéte *et al*. *(34)*, valine uptake is usually slow during the first ~12 h of fermentation, in which time the majority of the Group A amino acids are absorbed into the cell (Table 1). Romkes and Lewis *(36)* also observed in their study on the amino acid uptake rate by lager yeast harvested at stationary phase that valine was among the group of amino acids with the longest lag phase before absorption began. Kielland-Brandt *et al*. *(37)* also observed that valine uptake was slower in *Saccharomyces pastorianus* than in *Saccharomyces cerevisiae*, suggesting that valine could be classified as a Group C amino acid for *S. pastorianus* fermentations [as suggested by Palmqvist and Äyräpää *(33)*].

There are various membrane proteins responsible for the transport of valine into the yeast cell, including Gap1p (general amino acid permease 1), Bap2p (branched-chain amino acid permease 2), Bap3p (branched-chain amino acid permease 3) and Tat1p (main tyrosine transporter) *(38–40)*. The transport of amino acids into *S. cerevisiae* is active, and the amino acid permeases are driven by proton symport *(41)*. The transporters are not valine-specific, and other amino acids, especially the other branched-chain amino acids (leucine and isoleucine), also utilize the same transporters. Gap1p catalyses the transport of basic and neutral amino acids through the cell membrane, but it has a low affinity for branched-chain amino acids, and is down-regulated in the presence of preferred amino acids, such as glutamine and asparagine, and ammonia, suggesting it is only responsible for transporting a minor fraction of the valine in wort during fermentation *(42,43)*. Kodama *et al*. *(44)* studied the effect of constitutive expression and disruption of the Bap2p-encoding gene in *S. cerevisiae* on the valine uptake rate during fermentation, and observed that, with constitutive expression, the valine uptake rate during fermentation was increased, while disrupting the gene led to no significant changes in valine uptake rate. This confirmed that there are multiple transporters that transport valine into the cell during fermentation. Intracellular transport of valine (e.g. into the mitochondria) is not well understood, but it has been suggested that mitochondrial uptake of leucine occurs by active transport *(45)*.

Transcription of a Bap2p-encoding gene (Lg-*BAP2*, identical to Bap2p-encoding gene in *Saccharomyces bayanus*) in a lager yeast strain was repressed at the beginning of the fermentation and transcription was induced only when the majority of the amino acids in the wort were absorbed *(40)*. Similar results were observed by Gibson *et al*. *(46)* when they studied the amino acid uptake rates and expression of amino acid permease genes in an industrial lager yeast strain, as *BAP2* genes were expressed only towards the end of the fermentation. Kodama *et al*. *(40)* were not able to distinguish any specific compound or mechanism that induced transcription, but observed that leucine concentrations did not have any effect on transcription and that ethanol and organic acids repressed transcription. *BAP2* expression in *S. cerevisiae* is, on the other hand, induced primarily by the presence of leucine and only slightly by the presence of valine *(47)*. The transcriptional regulation of *BAP2* and other genes encoding branched-chain amino acid transporting permeases (*BAP3* and *TAT1*) is complex though, with several transcription factors, mainly the amino acid sensing Ssy1p protein, controlling the induced transcription of these genes *(48)*. Schoondermark-Stolk *et al*. *(49)* noticed that *BAP2* and *BAP3* genes were not transcribed in *S. cerevisiae* during fermentation of a supplemented YNB medium at pH 5, but they were transcribed during fermentation of the same medium adjusted to pH 3. Hence, the transcription of *BAP2* and *BAP3* during beer fermentation could be induced to some extent by the pH drop in wort during fermentation, causing an increase in valine uptake as fermentation progresses. Verbelen *et al*. *(50)* studied the effect of pre-oxygenating a lager yeast slurry pitching on the expression of various genes, including *BAP2*, during fermentation and concluded that the initial expression level (measured at 1 h after pitching) of *BAP2* was higher for a pre-oxygenated yeast compared with a non-pre-oxygenated yeast, but after 4.5 h the expression levels were similar. These results suggest that pre-oxygenation of the yeast before pitching could lead to a slight increase in initial valine uptake rates.

Process conditions and diacetyl formation

Since diacetyl is directly linked to the valine biosynthesis pathway, the intracellular valine concentration affects the amount of diacetyl generated during fermentation. It has been shown that valine strongly inhibits the acetohydroxyacid synthase (AHAS) enzyme, responsible for catalysing the formation of α-acetolactate from pyruvate (see Fig. 1) *(51,52)*. Hence, the more valine is present in the yeast cells, the less α-acetolactate will be synthesized, as the catalysing enzyme is inhibited, and consequently less diacetyl will be formed as well. Studies have shown varying data on the inhibitory effects of other branched-chain amino acids on AHAS. Both Barton and Slaughter *(52)* and

Magee and de Robichon-Szulmajster *(51)* observed that leucine inhibited the AHAS enzyme's activity, although not as much as valine. No inhibitory effect was observed with isoleucine. Pang and Duggleby *(53)* observed the opposite, that is, that isoleucine had a slight inhibitory effect and leucine had no inhibitory effect on AHAS activity.

Nakatani *et al*. *(54)* studied the effect of valine and isoleucine addition to fermenting wort on the production of diacetyl and found that increased wort valine concentrations significantly reduced the amount of diacetyl produced during fermentation. In fermentation trials with lager yeast involving wort of differing original gravities, free amino nitrogen and valine content, Petersen *et al*. *(55)* observed that low concentrations of valine in the wort resulted in the formation of double-peak diacetyl profiles (most likely as a result of valine depletion toward the end of fermentation), while high concentrations of valine in the wort resulted in single-peak diacetyl profiles with a lower maximum diacetyl level compared with the worts with low valine concentrations. The results show that the valine concentrations of the wort influence the amount of diacetyl formed, but the trials performed in the study varied in specific gravity and free amino nitrogen, meaning that no definite conclusions regarding the relationship between wort valine concentration and diacetyl concentration can be drawn. Cyr *et al*. *(56)* observed in trials with two different lager yeast strains that diacetyl concentrations in the fermenting wort were constant or decreased when valine uptake increased, while diacetyl concentrations increased when valine uptake decreased or was null. Krogerus and Gibson *(57)* showed that direct supplementation of wort with valine (100–300 ppm), and consequently greater uptake of valine by yeast cells, resulted in less diacetyl being formed during fermentation. Other fermentation parameters such as fermentation rate and yeast growth were unaffected *(57)*.

Maximum valine uptake for yeast cells pitched from rehydrated active dried yeast was up to 6 times lower than that of yeast cells pitched from a cropped fresh slurry, consequently causing a significantly higher diacetyl peak, indicating that the yeast drying process can invoke changes in the cell's membrane, influencing valine transport into the cell *(56)*. The rate of valine transport into yeast cells during fermentation could thus also influence the amount of diacetyl produced, since if the need for valine exceeds the transfer rate of valine into the cell, anabolic synthesis is required, potentially causing simultaneous diacetyl production. The valine uptake rate could conceivably be increased by lowering the ratio of leucine and isoleucine (since they utilize similar transporter proteins) to valine in the wort, leading to a potential decrease in diacetyl production as well. This would prove difficult in practical terms though, and could also increase the amount of 2,3-pentanedione produced. Romkes and Lewis *(36)* report that pre-conditioning harvested *S. cerevisiae* yeast in a glucose solution or by oxygenating decreases the length of lag phase before amino acid uptake, and hence valine uptake rate could potentially be increased by pre-treating the yeast in a glucose solution with aeration. These results also agree with those by Verbelen *et al*. *(50)*, who showed increased *BAP2* expression as a result of pre-oxygenating yeast before pitching. Valine addition to the pre-treatment solution and adjusting the pH of the pre-treatment to <5 could also potentially increase the valine uptake rate during fermentation and consequently decrease diacetyl production, since it increases the amount of branched-chain amino acid permease (Bap2p and Bap3p) genes transcribed *(49)*. Kodama *et al*. *(40)* on the other hand reported decreased expression of the *S.* (*eu*)*bayanus*-derived *BAP2* gene and no change of expression of the *S. cerevisiae*-derived *BAP2* gene in a lager yeast strain in YPM medium with 1 mM sorbate (pH 4.5) compared with the control YPM medium, suggesting that acidic conditions could have a negative effect on the production of branched-chain amino acid permeases.

The general FAN content of the wort may also affect the valine uptake rate and consequently diacetyl production. Krogerus and Gibson *(57)* reported that, when FAN levels were lowered, the diacetyl production was also lowered, presumably owing to faster absorption of preferred amino acids, resulting in an earlier and greater demand for valine and its increased uptake because of less competition for permease interactions. Increasing background levels of initial wort amino acids (while keeping valine concentration constant) resulted in a greater production of diacetyl. This increased production was influenced by which amino acids were increased. Preferred amino acids, that is, those taken up faster than valine, caused greater diacetyl formation in the first stage of fermentation, while increasing the concentrations of non-preferred amino acids influenced diacetyl levels later in the fermentation and therefore had a greater influence on the diacetyl levels in green beer *(57)*. Pugh *et al*. *(58)* also observed that the maximum diacetyl concentration during fermentation decreased as the initial FAN content was increased from 122 to 144 ppm, after which it again increased as the initial FAN content was increased from 144 via 168 to 216 ppm. Verbelen *(59)* reports a lower diacetyl production rate and simultaneously increased valine uptake rate and *BAP2* expression level in lager yeast for fermentations of 18°P worts containing adjuncts (FAN contents of around 150–210 ppm) compared with 18°P all-malt wort (FAN content around 300 ppm). Nakatani *et al*. *(54)*, on the other hand, report a negative correlation between the initial wort FAN content and the maximum VDK concentration observed during fermentation. These conflicting results are presumably owing to differences in valine uptake. At high FAN levels the yeast cell utilizes the preferred amino acids and less valine is taken up as a result (resulting in higher α-acetolactate production), while at very low FAN levels many amino acids will be entirely removed from the system and yeast growth is affected. If valine is depleted in this fashion then the demand for anabolic valine synthesis is increased and the α-acetolactate level increases as a result. It would appear from the values available in the literature that a FAN level of ~150 ppm is optimum for low diacetyl production; however, this value will vary depending on individual fermentation and process conditions. Lei *et al*. *(60)* also observed that the amount of valine absorbed during fermentation decreased when FAN content was increased from 264 ppm to 384, 398 and 433 ppm by adding protease enzymes during mashing, despite the increase in total valine concentration.

Barton and Slaughter *(52)* investigated the effect of adding individual amino acids and ammonium chloride in excess to wort on the VDK concentration and AHAS activity during fermentation, and found that alanine and ammonium chloride significantly lowered both the amount of diacetyl formed and the AHAS activity, suggesting they have an inhibiting effect on the enzyme. Valine and leucine also showed an inhibiting effect on AHAS (their effect on diacetyl concentration was not studied). The results suggest that alanine, ammonium chloride and possibly leucine could be used in excess together with valine in wort, to minimize the formation of diacetyl during fermentation, and that AHAS activity is vital for the control of diacetyl formation. Dasari and Kölling *(61)* observed elevated diacetyl production in petite

mutants of *S. cerevisiae*, as a result of cytosolic localization of the AHAS enzyme, suggesting that accumulation of AHAS in the cytosol could result in increased diacetyl production, possibly as a result of increased secretion of α-acetolactate from the cell.

Pitching rate and cell density also affect the amount of diacetyl present in beer at the end of fermentation, as it has been observed that concentration increases with increased pitching rate. In trials with various lager yeast strains, Verbelen *et al. (62)* observed over 10-fold increases in beer diacetyl concentrations when pitching rate was increased several-fold *(62,63)*. This can be explained by the fact that more α-acetolactate was presumably produced and fermentation times were shorter at higher pitching rates, reducing the amount of α-acetolactate spontaneously decarboxylated to diacetyl outside the cells during active fermentation in the rate-limiting step of the diacetyl removal pathway, leading to increased post-fermentation α-acetolactate and eventually diacetyl concentrations. On the other hand, Erten *et al. (64)* observed decreasing concentrations of diacetyl with increasing pitching rates (10×10^6 viable cells/mL to 100×10^6 viable cells/mL) in beer fermented from high-gravity wort with lager yeast. In this study all the fermentations were carried out for 11 days though, while the fermentations carried out by Verbelen *et al. (62,63)* lasted for ~2–14 days depending on strain and pitching rate, with shorter fermentations times at higher pitching rates. This shows a negative correlation between the fermentation time and diacetyl concentration in the beer, with the yeast having more time to assimilate and reduce the diacetyl the longer it is in contact with the beer. Ekberg *et al. (65)* also observed increased concentrations of VDK in beer fermented for 72 h with a stress-tolerant strain, compared with beer fermented for 193 h with the control strain. Sigler *et al. (66)* observed increased diacetyl production with increased wort osmolarity (adjusted with sorbitol), most likely as a result of a decrease in yeast vitality.

Diacetyl may also be formed in packaged beer during beer ageing, as a result of Maillard reactions or oxidation of acetoin and 2,3-butanediol *(67,68)*. This formation of diacetyl is especially relevant at elevated temperatures and dissolved oxygen concentrations during ageing. It has also been found that diacetyl may be formed during re-fermentation of beer in bottle-conditioned beer owing to yeast growth *(69)*. However, in the same study it was observed that diacetyl concentrations were eventually decreased to below the initial concentration as a result of re-fermentation.

The valine content and amino acid profile of wort could potentially be altered by modifying mashing conditions, since amino acids are released into the wort through the action of a large variety of proteolytic enzymes in the malt during the mash *(70)*. By mashing under conditions favouring the activity of those enzymes releasing more valine into the wort, diacetyl formation during fermentation could be lowered. Schwarz *et al. (71)* investigated the effect of various 'mashing-in' (conditions at the beginning of the mash) temperatures, pH values and times on the concentration of several amino acids in the wort, and results show that a 'mashing-in' temperature of 50 °C, a pH of 5.4 and time of 60–75 min seem to be optimal conditions for a high wort valine concentration and a high ratio of valine to other branched-chain amino acids. Gómez Guerrero *(72)* also reported that the valine content of wort increased in the beginning of the mash at increased time at 48 °C, but decreased as the temperature of the mash was increased to 65 °C. Hence, the valine content of the wort, as well as ratio of valine to other amino acids, could be increased through the use of optimum mashing conditions.

The type of malt used for producing the wort also has an effect on the amino acid profile of the wort, since proteolytic enzymes are active during the malting process (specifically during germination) as well, while high kilning temperature can decrease the concentration of amino acids in the malt *(70)*. Samaras *et al. (73)* studied the effect of using various malt types on the amino acid content of the wort (see Table 2), and found that malts kilned at lower temperatures contained a higher concentration of both valine and the total amount of amino acids. The cereal grain used for producing the malt could also affect the amino acid profile of the wort, since different cereal grains contain different amounts and types of protein (Table 3). Oat protein has a higher valine content than barley protein, suggesting that wort produced from oat malt might have a higher valine concentration than wort produced from barley malt. Klose *et al. (74)* showed that brewing a 100% oat malt beer is possible, but extract yield was decreased and wort viscosity increased compared with wort produced from barley malt. Also, diacetyl concentration at the end of fermentation was higher for the beer brewed from oat malt, compared with the one brewed from barley malt, despite its increased valine content, suggesting that other wort parameters (e.g. FAN content and pH) have more effect on diacetyl formation.

Valine supplementation to wort or modification of the wort amino acid profile could also potentially result in unwanted side-effects, such as an increased production and concentration of higher alcohols. In *Saccharomyces* spp., higher alcohols (or fusel alcohols) are formed through the catabolism of certain amino acids, for example, branched-chain amino acids via the Ehrlich pathway *(29)*. Valine is converted into isobutanol through a transamination, decarboxylation and oxidation reaction. Isobutanol has

Table 3. The valine content (percentage of total protein) of various cereal grains

Cereal	Valine content	Source
Barley	4.7–4.9	*(121–123)*
Oats	5.0–5.7	*(121–124)*
Rye	4.4–4.8	*(122,123)*
Wheat	4.4	*(121–123)*

Table 2. The amino acid content (mg per 100 g of malt) of worts prepared with various malts *(73)*

Amino acid	Green malt	Lager malt	Pale malt	Cara malt	Crystal malt	Black malt	Chocolate malt
Valine	6.7	7.3	3.1	2.7	1.6	0.5	0.5
Leucine	8.0	8.4	4.19	1.6	0.5	0.5	0.5
Isoleucine	4.0	4.2	2.1	0.5	0.5	0.5	0.5
Total	111.0	146.9	61.8	38.9	17.3	6.2	4.1

a flavour threshold of around 200 ppm in beer and it has an alcoholic and solvent-like flavour *(1)*. Over-supplementation of valine to wort could thus lead to increased concentrations of isobutanol in the beer and potential off-flavours. Valine itself has a flavour threshold of around 400 ppm *(75,76)* and it has bitter-sweet flavour *(77)*. Valine concentrations in both unfermented wort and beer are typically much lower than the flavour threshold, but by supplementing valine to the wort, concentrations above the flavour threshold of valine in the wort can easily be reached. A fraction of this valine, which depends on yeast strain and concentrations of other amino acids in the wort, is absorbed by yeast during fermentation, but the rest remains in the wort and may cause off-flavours. Amino acids may also undergo Strecker degradation into aldehydes during beer ageing when oxygen is present, suggesting that the long-term stability of amino acid-supplemented beer may be lessened *(78)*. Costs of valine supplementation, in whatever form, to wort must be less than the costs saved from a decreased maturation time if the technique is to be profitable for a brewery.

Process conditions and diacetyl reduction

Yeast is capable of assimilating and reducing the VDK formed into diols, which have much higher flavour thresholds compared with VDK, and hence do not affect the flavour and aroma of the beer. Diacetyl is ultimately reduced to 2,3-butanediol, which has a flavour threshold of around 4500 ppm *(1,79)*. In *Saccharomyces* spp., diacetyl is reduced to 2,3-butanediol via acetoin, and a number of different (both specific and non-specific, as well as NADH- or NADPH-dependent) ketone reductase enzymes have been identified in various yeast strains *(80–83)*. In a study of ale and lager yeast strains, Murphy *et al*. *(84)* observed a specific acetoin reductase enzyme in lager strains not present in ale strains, which expressed a dehydrogenase enzyme active on both diacetyl and acetoin. Bamforth and Kanauchi *(80)* also observed a greater number of diacetyl reductase enzymes in lager yeast compared with ale yeast. The *ADH1*-encoded alcohol dehydrogenase, mainly responsible for the conversion of acetaldehyde into ethanol, has been shown to also reduce diacetyl *(85)*.

The removal of diacetyl in the later stages of fermentation is not as well understood as the formation of diacetyl. The removal of diacetyl from beer by the yeast is rapid, as shown by Boulton and Box *(86)* and Inoue and Yamamoto *(87)*, where diacetyl addition to active fermentations resulted in diacetyl peaks rapidly declining back to the diacetyl levels of the control fermentation. These results suggest that diacetyl reduction by yeast is not a rate-limiting factor in diacetyl removal, but rather the spontaneous decarboxylation of α-acetolactate to diacetyl. However, in the same study, the width of the diacetyl peak after diacetyl addition increased when the additional diacetyl was introduced later in the fermentation, suggesting that yeast physiological condition does have some influence on the rate of diacetyl reduction, most likely by affecting the assimilation rate of diacetyl into the cell. The exact mechanisms of diacetyl uptake into yeast are not known, but the diacetyl uptake rate into yeast cells and consequently the diacetyl removal rate has been shown to be affected by fermentation parameters influencing yeast membrane composition (such as temperature and oxygenation of yeast or wort), while any effects will be amplified by phenomena such as yeast flocculation and sedimentation *(86)*.

The pH of the wort and the fermentation temperature also influence the amount and rate of diacetyl formed and reduced, as they affect yeast growth rate (and consequently the amount of branched-chain amino acids biosynthesized), the reaction rate of the spontaneous decarboxylation of α-acetolactate into diacetyl, and the activities of the enzymes responsible for reducing diacetyl to acetoin and 2,3-butanediol *(35)*. Increased fermentation temperatures lead to higher initial diacetyl production rates as a consequence of increased yeast growth, but also produce more yeast mass to reduce the diacetyl to 2,3-butanediol and increase the reaction rate of the oxidative decarboxylation of α-acetolactate to diacetyl, which suggests that the rate-limiting conversion of α-acetolactate to diacetyl is expedited at higher temperatures, ultimately leading to sharper diacetyl concentration peaks during fermentation and thus a faster diacetyl reduction rate *(2,35,88)*. The increased decarboxylation rate of α-acetolactate to diacetyl at higher temperatures can be exploited during fermentation in a so-called 'diacetyl rest', where temperatures are increased towards the end of fermentation in order to more rapidly decrease wort α-acetolactate concentrations and shorten the maturation period. Bamforth and Kanauchi *(80)* report an optimum pH of 3.5 for an acetoin dehydrogenase enzyme isolated from a commercial lager yeast strain, suggesting that diacetyl reduction rates are higher at lower wort pH values (i.e. towards the end of fermentation). Several sources *(21,35,89)* report an increased reaction rate for the oxidative decarboxylation of α-acetolactate to diacetyl at lower pH values, which also suggests that the rate-limiting conversion of α-acetolactate to diacetyl is faster at more acidic wort conditions. The maturation time needed for diacetyl reduction could thus be reduced at lower beer pH values, as long as the pH stays within the range that is suitable for a palatable beer. The pH of beer is typically in the range of 4.0–4.5 but can vary from around 3.7 to 5.0 depending on style and ingredients *(90)*.

The reduction of diacetyl in beer that has finished fermentation can be speeded up with the use of immobilized yeast cells *(91,92)*. By passing fermented beer to a packed bed reactor containing immobilized yeast cells after heat treatment (to increase conversion of α-acetolactate to diacetyl and acetoin) or enzyme treatment (by immobilized α-acetolactate decarboxylase), VDK concentrations and maturation times can be rapidly decreased. Lower VDK concentrations have also been observed during continuous primary fermentation in an immobilized yeast system compared with batch fermentations, as a result of decreased yeast growth and simultaneous need for BCAA synthesis *(93)*. This reduction method, like the control of the wort amino acid profile, requires no use of genetically engineered yeast, and is thus in commercial use. However, the use of an immobilized yeast cell reactor requires a significant investment by the brewery.

Strain development

Other strategies for diacetyl control in beer include reducing the activity of, or disrupting the yeast genes coding for, the AHAS enzyme, responsible for catalysing the formation of α-acetolactate from pyruvate (see Fig. 1), increasing the metabolic flux through the pathway from α-acetolactate to valine, and exposing the fermenting wort to, or introducing into yeast the genes coding for, the α-acetolactate decarboxylase enzyme, which catalyses the non-oxidative decarboxylation of α-acetolactate into acetoin. The use of genetically engineered yeast strains in commercial beer is not yet possible though, owing to a lack of consumer

acceptance and legal regulations *(94)*. Wang *et al*. *(95)* report that diacetyl production was decreased by 64 and 58% in beer fermentations with two modified yeast strains [disrupted AHAS-coding gene (*ILV2*) and integrated γ-glutamylcysteine synthetase-coding gene (*GSH1*)] compared with fermentations with the unmodified strains. The disruption caused lower expression of AHAS and consequently less diacetyl was formed. Liu *et al*. *(96)* also observed decreased diacetyl production (66%) and shorter maturation times with the disruption of *ILV2* in an industrial strain of *S. pastorianus*.

Feedback inhibition of the *ILV2*-encoded AHAS enzyme in *S. cerevisiae* is most likely achieved by the action of a regulatory subunit, encoded by the *ILV6* (*YCL009c*) gene. Cullin *et al*. *(97)* observed that the inhibiting effect of valine on AHAS activity in *S. cerevisiae* was lost with the deletion of *ILV6*. Similar results were noticed by Pang and Duggleby *(98)* when they overexpressed *ILV2* and *ILV6* genes from *S. cerevisiae* in *Escherichia coli*. The inhibiting effect of valine on AHAS activity was greater when both *ILV2* and *ILV6* were expressed, compared with when only *ILV2* was expressed. In the presence of no supplemented valine, Pang and Duggleby also observed lower AHAS activity when only *ILV2* was expressed, compared with when both *ILV2* and *ILV6* were expressed, suggesting that the *ILV6*-encoded subunit not only is responsible for feedback inhibition, but also enhances the general activity of AHAS. Cullin *et al*. *(97)* on the other hand, did not observe any decrease of AHAS activity with the deletion of *ILV6*, but Pang and Duggleby *(98)* showed that the enhancing effect of the *ILV6*-encoded subunit is highly dependent on environmental conditions, such as phosphate concentration and pH. Duong *et al*. *(99)* found lower expression levels of *ILV6* during fermentation in a lager yeast strain with low diacetyl production compared with other yeast strains with higher diacetyl production rates. They also observed lower diacetyl production rates during fermentation for a lager yeast strain with a disrupted *Sc-ILV6* gene compared with the unmodified strain, suggesting that the *ILV6*-encoded subunit enhances the activity of the *ILV2*-encoded AHAS enzyme. Hence, the amount of α-acetolactate produced during fermentation could potentially be decreased if the expression of *ILV6* was reduced, but on the other hand, the inhibiting effect of valine, and perhaps other branched-chain amino acids, would be lost as well.

The amount of α-acetolactate leaking out of the yeast cells could potentially be decreased by increasing the flow of α-acetolactate and the other intermediates in the pathway to valine (see Fig. 1). This can be achieved by increasing the activities and expression of the enzymes catalysing the reactions in the pathway. The acetohydroxyacid reductoisomerase enzyme, encoded by the *ILV5* gene, is responsible for the conversion of α-acetolactate to 2,3-dihydroxy-isovalerate in *Saccharomyces* yeasts *(100)*. Dillemans *et al*. *(18)* examined the effect of introducing an *ILV5* gene coding for a highly active acetohydroxyacid reductoisomerase enzyme from a mutant *S. cerevisiae* strain into an FL10 *S. cerevisiae* strain on the amount of VDK produced during growth on a minimal medium, and concluded that the amount of VDK produced by the recombinant strain was reduced by 50–60% compared with the control strain and therefore the reaction catalysed by acetohydroxyacid reductoisomerase can be seen as rate-limiting in the pathway from α-acetolactate to valine. Similar results were obtained by Mithieux and Weiss *(101)*, with the introduction of *ILV5* genes (on plasmids) to lager yeast, resulting in the expression of more acetohydroxyacid reductoisomerase and less diacetyl formation. Kusunoki and Ogata *(102)* also successfully integrated copies of *ILV5* genes upstream of the *S. cerevisiae*-type *ILV2* gene in an *S. pastorianus* strain, as well as replaced *ILV2* genes with a similarly sequenced *SMR1B* gene (sulfometuron methyl resistance gene), to construct a lager yeast strain that both produced significantly lower amounts of VDK (60% reduction) during fermentation and reduced the amount of diacetyl to below the flavour threshold much more rapidly (67 h compared with 160 h) compared with the control strain. Lu *et al*. *(103)* also observed less diacetyl production throughout fermentation with a lager yeast strain modified for *ILV5* overexpression compared with the unmodified strain. Bussey and Umbarger *(104)* tested the effect of supplementation of various amino acids on the specific activities of enzymes involved in the valine biosynthesis pathway in a *Saccharomyces* sp., and found that the reductoisomerase, encoded by the *ILV5* gene, was inhibited by the presence of valine, leucine and threonine. Bollon and Magee *(105)* showed that threonine deaminase, responsible for catalysing the conversion of threonine to α-ketobutyrate in the first step of the isoleucine biosynthesis pathway (see Fig. 1), also functions as a regulatory protein for the other enzymes involved in the valine and isoleucine biosynthesis pathways, and that regulation is dependent on the presence of valine, leucine and isoleucine.

During the fermentation of glucose to ethanol, two molecules of NADH are produced together with every molecule of ethanol. The *ILV5*-encoded acetohydroxyacid reductoisomerase enzyme, responsible for converting α-acetolactate into 2,3-dihydroxy-isovalerate in the valine biosynthesis pathway (see Fig. 1), is NADPH-dependent *(100)*. Increased flux towards valine, resulting in less α-acetolactate being secreted out of the cell, could potentially be achieved by modifying the *ILV5*-encoded enzyme to utilize NADH as well or by adjusting the redox balance by converting NADH to NADPH through the overexpression of a NADH kinase (such as the one encoded by *POS5*). Hasegawa *et al*. *(106)* report an increased yield of valine from glucose in fermentation with *Corynebacterium glutamicum* expressing an *ilvC*-encoded acetohydroxyacid reductoisomerase with a modified cofactor requirement (from NADPH to NADH). Anthony *et al*. *(107)* report increased isobutanol (produced from valine or valine biosynthesis precursors) yields from pyruvate in *S. cerevisiae* overexpressing the *POS5* gene. Similar strategies with *Saccharomyces* spp. could reduce diacetyl production during beer fermentation, but the fermentation rate of the yeast and flavour profile of the beer could be affected by metabolic changes caused by changes in the cofactor balance.

The use of targeted genetic modification may be circumvented through the use of adaptive evolution. This approach has been used to modify beer and sake brewing yeast strains for greater tolerance to fermentation-associated stresses *(65,108–110)* and for modified production of flavour compounds *(111,112)*. Gjermansen *et al*. *(113)* have produced lager strain variants with lowered diacetyl production by selecting for resistance to the herbicide sulfometuron methyl, which inhibits acetohydroxyacid synthase. Isolated variants produce as little as half the level of diacetyl produced by the parental strain owing to altered AHAS activity. Using this technique it was possible to obtain a variant that produced beer of superior quality to the parental strain (a direct result of reduced diacetyl level) *(113)*.

Owing to the build-up and leaking of α-acetolactate and since the subsequent reactions to diacetyl or 2,3-dihydroxy-isovalerate are rate-limiting in the diacetyl-valine cycle, another approach for minimizing diacetyl formation is the conversion of α-acetolactate directly to compounds with higher flavour thresholds, such as acetoin. The α-acetolactate decarboxylase enzyme

Table 4. Methods for decreasing residual diacetyl concentrations during the fermentation of beer

Method	GMO	Mechanism	Disadvantages
Increased conditioning time[a]		Increased contact time between the yeast cells and the beer allows for more reduction of diacetyl to 2,3-butanediol by the yeast.	Increases production time.
Decreased pH of beer[b]		Increased reaction rate for the oxidative decarboxylation of α-acetolactate to diacetyl.	pH affects the flavour and mouthfeel of beer.
Increasing fermentation and/or maturation temperature (diacetyl rest)[c]		Increased fermentation temperatures result in increased diacetyl formation, but also produce more yeast for diacetyl reduction and increase the reaction rate for the oxidative decarboxylation of α-acetolactate to diacetyl.	Fermentation temperature affects fermentation performance and the flavour profile of the beer.
Valine supplementation[d]		Increased wort valine concentrations increase the transport of valine into the cell and decrease the activity of the acetohydroxyacid synthase enzyme, resulting in less α-acetolactate formed.	Costly. Potential increase in higher alcohols and esters.
Optimizing wort FAN content[e]		A low wort FAN content can result in increased valine uptake rates but decreased valine concentrations, while a high wort FAN content results in decreased valine uptake rates but an increased valine concentration.	A low FAN content can affect yeast health and growth rate, while a high FAN content can lead to a potential increase in higher alcohols and esters, and can increase growth of spoilage microbes.
Disrupting *ILV2* or *ILV6*[f]	×	Less or no α-acetolactate is formed by the cell, resulting in less diacetyl in the beer.	GMO. May affect yeast health and growth rate, if not enough valine, isoleucine and leucine is available.
Overexpressing *ILV5* and/or *ILV3*[g]	×	Increases the flux through the valine biosynthesis pathway, resulting in less α-acetolactate being secreted out of the cell.	GMO. Potential increase in higher alcohols and esters.
Introduction and expression of ALDC-encoding gene[h]	×	The α-acetolactate decarboxylase enzyme catalyses the non-oxidative decarboxylation of α-acetolactate into acetoin, resulting in less diacetyl in the beer. The yeast cells would produce the enzyme themselves, meaning no supplementation would be required.	GMO. May affect yeast health and growth rate, if not enough valine, isoleucine and leucine are available.
Supplementing ALDC enzyme[i]		See above.	Costly if added in batches, and requires considerable investment if encapsulated enzymes are used.
Using immobilized yeast cells[j]		Passing beer through immobilized yeast allows for rapid reduction of diacetyl to 2,3-butanediol by the yeast.	Investment costs. Potential sensory changes of the beer. Requires pre-conversion (e.g. enzymatically or by heat) of α-acetolactate to diacetyl.
Optimizing mash conditions[k]		The use of mash temperatures, pH and times that promote the release of valine into the wort can increase the initial valine concentration, and ratio of valine to other amino acids, in the wort. See mechanism for 'valine supplementation'.	Potential increase in production time and decrease of mash efficiency.
Optimizing malting conditions[l]		The amount of free valine in the malt can be affected by the malting conditions (temperature and times), potentially resulting in increased valine concentration in	Activity of the various cytolytic, proteolytic and amylolytic enzymes in the malt may be affected, resulting in decreased extract yields and modified wort properties.

(Continues)

Table 4. (Continued)

Method	GMO	Mechanism	Disadvantages
		wort after the mash. See mechanism for 'valine supplementation'.	
Quality and type of malt[m]		The protein amount and quality of the cereal grains used for producing malt depend highly on growth conditions, soil quality and species. Using grains high in valine for producing malt could potentially result in increased valine concentrations in wort after the mash. See mechanism for 'valine supplementation'.	May decrease extract yield and affect wort properties. Recipes are formulated for a certain malt type, so changing malt types can affect sensory properties of the beer.

[a](80); [b](21,35,80,89); [c](2,35,88); [d](49,51,55–57); [e](57–59); [f](95,96,99,102); [g](18,101–103); [h](116–119); [i](114,115); [j](91–93); [k](70–72); [l](70,73); [m](74,121–124).

(ALDC), which catalyses the non-oxidative decarboxylation of α-acetolactate into acetoin, is expressed by a variety of bacteria, and attempts have been made to reduce diacetyl concentrations in beer by both adding the enzyme directly to the fermenting beer *(114–116)* and by introducing the gene encoding for the enzyme to brewer's yeast *(117–120)*. The studies have shown that diacetyl concentrations during fermentation are lowered in the presence of an α-acetolactate decarboxylase enzyme and the length of any maturing period (or secondary fermentation) is significantly shortened. ALDC enzymes are commercially available for brewing purposes, but the problem regarding the use of genetically engineered yeast strains and the use of foreign DNA remains, so ALDC-producing yeast strains are therefore not used commercially.

The various methods for decreasing the amount of residual diacetyl produced during wort fermentation are presented in Table 4. As can be seen from the table, there exists a wide variety of techniques that brewers can utilize to minimize diacetyl production during fermentation and simultaneously reduce the maturation time needed for the beer. The listed techniques rely mainly on one of two different approaches for diacetyl control, either minimizing or preventing the formation of α-acetolactate, or enhancing the removal of α-acetolactate or diacetyl formed.

Conclusion

Since diacetyl is typically unwanted in beer, its removal is one of the main objectives of beer maturation, especially for lager beer, and the maturation phase constitutes a time- and resource-consuming step in the overall production process. Diacetyl production is strongly linked to the cell's need for valine, and any changes in process conditions that increase the need for intracellular biosynthesis of valine (e.g. those that stimulate yeast growth or reduce valine uptake) will result in increased diacetyl production. The removal of diacetyl from beer is affected mainly by the rate of spontaneous decarboxylation of α-acetolactate to diacetyl, while the reduction of diacetyl is carried out by the yeast. The process of diacetyl reduction by yeast is not as well understood as the process of formation, but is most likely dependent on factors such as physiological condition, membrane composition, temperature and pH. As a result, several approaches for minimizing the formation of diacetyl during fermentation and its presence in beer have been identified. In recent years, a wide range of strategies for the production of low-diacetyl yeast strains through genetic engineering have emerged, but commercial use with these strains is currently not practised. Cool storage temperatures, limited oxygen presence and sanitary practices will counter the formation of diacetyl in packaged beer. Much research has been conducted on understanding diacetyl formation and reducing diacetyl production, but the area still remains a challenge, especially with regard to new brewing technologies, such as continuous fermentation, high-gravity brewing and in particular any process change that results in a shorter fermentation time.

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