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CONTRIBUTION OF WORT VALINE TO DIACETYL LEVELS IN WORT DURING BREWERY FERMENTATION

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Abstract

Diacetyl and 2,3-pentanedione are butter-tasting vicinal diketones produced as by-products of valine and isoleucine biosynthesis during wort fermentation, and their presence in beer is usually unwanted. Yeast are able to reduce diacetyl and 2,3-pentanedione into compounds with higher flavour thresholds, and this is usually achieved during a resource- and time-consuming maturation phase after the primary fermentation. One promising method of decreasing diacetyl production during fermentation is through the control of the valine content of the wort, since valine is involved with the feedback inhibition of enzymes controlling the valine biosynthesis pathway and indirectly the formation of diacetyl precursors. The objective of this thesis was to investigate the influence of valine supplementation, wort amino acid profile and free amino nitrogen content on diacetyl formation during wort fermentation with the lager yeast *Saccharomyces pastorianus*, by carrying out a series of small-scale (2-litre) and medium-scale (10-litre) fermentations, which were sampled and analysed for diacetyl and amino acid concentrations, along with standard beer parameters.

Supplementation of valine to the wort resulted in decreased maximum (up to 37% lower) and final (up to 33% lower) diacetyl concentrations in all trials, and the composition of the amino acid spectrum of the wort also had an impact on diacetyl and 2,3-pentanedione production during fermentation. The results suggest that there is no correlation between the concentrations of wort amino acids and diacetyl production per se, but rather a negative correlation between the uptake rate of valine (and also other branched-chain amino acids) and diacetyl production was found. The results from all four experiments agree that fermentation performance and yeast growth was not affected by the studied levels of amino acid supplementations, implying that supplementation of valine or other amino acids will not affect the fermentation time nor the attenuation level achieved. Supplementation of amino acids did however have a minor effect on the concentrations of higher alcohols and esters linked to the amino acids via the Ehrlich pathway, suggesting that large levels of supplementation could affect the flavour profile of the beer.

It can be concluded that modifying the amino acid profile of wort, especially in respect to valine and the other branched-chain amino acids, is an effective way of decreasing the amount of diacetyl formed during fermentation. This knowledge can be utilized for decreasing the maturation time of beer, however further research is required before it becomes feasible at an industrial scale.

Keywords Diacetyl, valine, amino acid, beer, yeast, fermentation

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Diacetyl och 2,3-pentandion är smörsmakande vicinala diketoner som produceras som biprodukter av valin- och isoleucinbiosyntes i jäst under jäsningen av vört, och dess förekomst i öl är oftast oönskad. Jäst kan även reducera diacetyl och 2,3-pentandion till ämnen med högre smaktröskel, och detta åstadkoms i en resurs- och tidskrävande mognadsfas efter primärjäsningen. En lovande metod för att minska diacetylproduktionen under jäsningen är genom att justera valinhalten i vörten, eftersom valin hämnar diverse enzymer involverade i valinbiosyntesen och därmed indirekt minskar mängden diacetylföregångare som produceras. Målet med detta diplomarbete var att undersöka påverkan av valintillsättning, vörtens aminosyraprofil och fria kväve på diacetylproduktionen under jäsningen av vört med lagerjästen *Saccharomyces pastorianus*. Detta utfördes genom småskaliga (2 liter) och mellanskaliga (10 liter) jäsningar, som samplades och analyserades för diacetyl- och aminosyrakoncentrationer, tillsammans med standard ölparametrar.

Tillsättningen av valin till vörten resulterade i minskade maximala diacetylkoncentrationer (upp till 37% mindre med de undersökta tillsättningsnivåerna) och diacetylkoncentrationer i slutet av jäsningen (upp till 33% mindre med de undersökta tillsättningsnivåerna) i alla försöken. Kompositionen av vörtens aminosyraprofil visade sig även ha en betydande inverkan på både diacetyl- och 2,3-pentadionproduktionen under jäsningen. Resultaten tyder på att det inte finns någon direkt korrelation mellan koncentrationerna av aminosyror och diacetylproduktion, utan snarare påträffades en negativ korrelation mellan valinets (och övriga grenade aminosyror) upptagningshastighet och diacetylproduktionen. Resultaten från alla fyra experiment är överens med att jäsningsprestandan och jästtillväxten inte påverkas av de undersökta tillsättningsnivåerna, vilket tyder på att tillsättning av valin eller andra aminosyror varken påverkar jäsningstiden eller -graden. Tillsättningen av aminosyror visade sig dock ha en mindre effekt på koncentrationen av högre alkoholer och estrar länkade till aminosyror genom Ehrlichmekanismen, vilket tyder på att höga tillsättningsnivåer kan inverka på ölets smakprofil.

Därmed visar resultaten att modifiering av vörtens aminosyraprofil, speciellt med avseende på valin och övriga grenade aminosyror, är en effektiv metod att minska mängden diacetyl som bildas under jäsningen. Denna kunskap kan utnyttjas för att minska mognadstiden för öl, men vidare forskning krävs ännu för att den ska kunna utnyttjas i industriell skala.

Nyckelord diacetyl, valin, aminosyra, öl, jäst, jäsning

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22nd of March 2013,
Espoo, Finland

Kristoffer Krogerus

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Abbreviations

AHAS	Acetohydroxyacid synthase (EC 2.2.1.6)
ALDC	Acetolactate decarboxylase (EC 4.1.1.5)
BAP2	Gene encoding branched-chain amino acid permease 2
Bap2p	Branched-chain amino acid permease 2
BAP3	Gene encoding branched-chain amino acid permease 3
Bap3p	Branched-chain amino acid permease 3
BAT1	Gene encoding branched-chain amino acid aminotransferase 1
BAT2	Gene encoding branched-chain amino acid aminotransferase 2
BCAA	Branched-chain amino acid
FAN	Free amino nitrogen
GAP1	Gene encoding general amino acid permease 1
Gap1p	General amino acid permease
GSH1	Gene encoding γ-glutamylcysteine synthetase
ILV2	Gene encoding acetohydroxyacid synthase
ILV5	Gene encoding acetohydroxyacid reductoisomerase
ILV6	Gene encoding regulatory subunit of acetohydroxyacid synthase
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NPAA	Non-preferred amino acid
PAA	Preferred amino acid
POS5	Gene encoding NADH kinase
TAT1	Gene encoding tyrosine and tryptophan amino acid transporter 1
Tat1p	Tyrosine and tryptophan amino acid transporter 1
VDK	Vicinal diketone
YNB	Yeast nitrogen base medium
YPM	Yeast extract-, peptone-, and maltose-based growth medium

1 Introduction

Beer or beer-like beverages have been produced for thousands of years, with the earliest evidence tracing as far back as 7000 BC in Ancient Babylonia, and they are consequently one of the oldest fermented beverages known to man. During the majority of its history, brewing was a process performed domestically or on a small-scale commercial basis, with wild yeasts and whatever local ingredients were obtainable, but after the industrial revolution in the 19th century the brewing process became industrialized, and now a few companies worldwide produce the majority of the beer consumed today. Although the actual brewing process used today remains essentially traditional and similar to the one used prior to industrialization, new knowledge, techniques and innovations regarding the brewing process are frequently being introduced. (Jay *et al.*, 2005; Meussdoerffer, 2009)

Diacetyl and 2,3-pentanedione (also known as vicinal diketones) are produced by the yeast during fermentation as by-products of valine, leucine and isoleucine biosynthesis. Vicinal diketones are usually unwanted in finished beer because of their butter-like flavour and low flavour thresholds. In addition to producing VDK precursors, yeast cells are able to reduce diacetyl and 2,3-pentanedione to acetoin, 2,3-butanediol and 2,3-pentanediol, which in turn have much higher flavour thresholds, and rarely influence the sensory properties of the beer. Hence, one of the main purposes of beer maturation is the lowering of VDK concentrations. This step in the production process is time-consuming, and it is of interest for the breweries to decrease this maturation time, without affecting the quality of the final beer. Research has been conducted on understanding diacetyl formation and reducing diacetyl production, but the area still remains a challenge, especially in regard to new brewing technologies, such as continuous fermentation and high gravity brewing. (Boulton and Quain, 2001)

One promising method of decreasing diacetyl production during fermentation is through the control of the valine content of the wort. Valine is involved with the feedback inhibition of enzymes controlling the valine biosynthesis pathway, and thus

simultaneously involved in the control of the formation of diacetyl precursors (Magee and de Robichon-Szulmajster, 1968). The concentrations of other amino acids (besides valine) in the wort may also indirectly affect diacetyl production, since they affect the uptake rate of valine into the cell and may also be involved in enzyme inhibition. Hence, the objective of this thesis was to investigate the influence of valine supplementation, wort amino acid profile and free amino nitrogen content on diacetyl formation during wort fermentation with the lager yeast *Saccharomyces pastorianus*. This was accomplished by carrying out a series of small-scale (2-litre) and medium-scale (10-litre) fermentations, which were sampled and analysed for diacetyl and amino acid concentrations, along with standard beer parameters. The beer resulting from the medium-scale fermentations was also processed, bottled and analysed, to examine the effects of the variables on the end product quality. The thesis begins with a literature review on the subject, after which the experimental fermentations and the results are described.

2 Literature review

2.1 Overview of the beer production process

Beer is produced mainly from malted cereal grains, hops, yeast and water. The beer production process involves four major stages: malting, wort production, fermentation and down-stream processing. Malting is the steeping, germination and kilning of cereal grains to produce malt. These malts are then mashed, to convert the starch they contain into fermentable sugars. The liquid extracted from the mash, i.e. the wort, is then boiled together with hops and possible adjuncts. After the boil, the wort is cooled and any solid matter is removed. Yeast is then added to the wort, and fermentation begins. After fermentation, the beer is matured and yeast is harvested. After maturation the beer undergoes processing (e.g. filtration, stabilization and bottling) into the final product. A simplified diagram depicting the brewing process of a lager beer is presented in Figure 1. (Briggs *et al.*, 2004; Linko *et al.*, 1998)

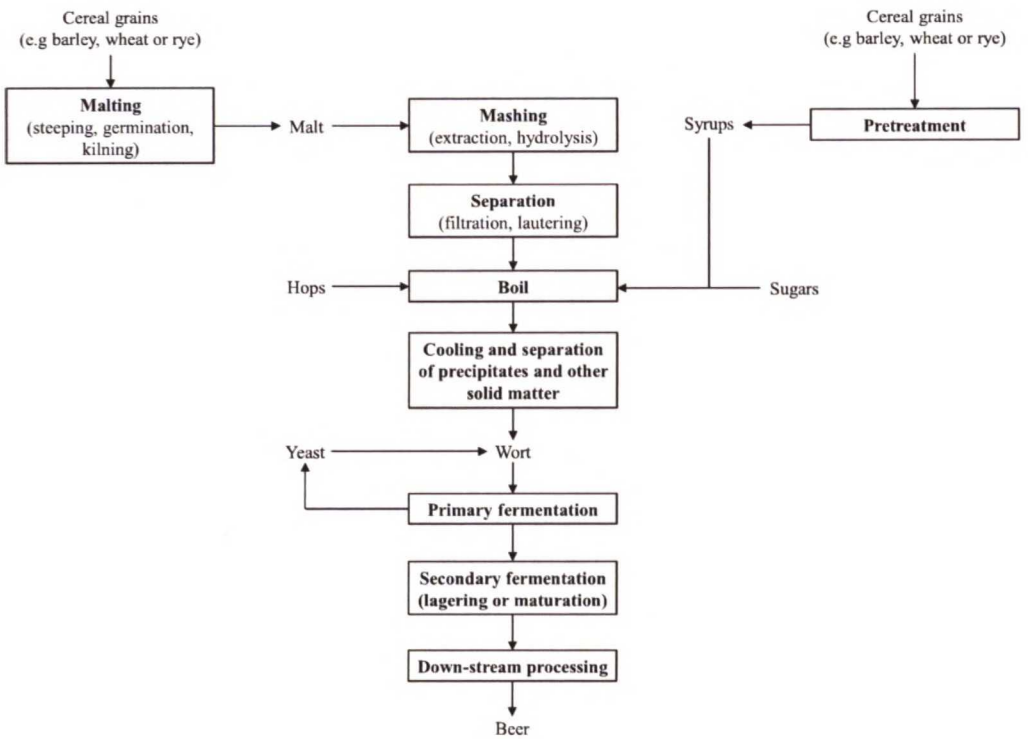

Figure 1 – A simplified diagram of the brewing process. Boxes indicate processes, while plain text indicates raw materials or products. (Linko *et al.*, 1998)

2.1.1 Malting

Malted cereals are the main sugar sources for fermentation in beer, though raw cereal grains and adjuncts, such as sugars and syrups, are used as well. The main cereal grain used for brewing is barley (*Hordeum vulgare*), but wheat (*Triticum aestivum*), sorghum (*Sorghum vulgare*), rye (*Secale cereale*) and oats (*Avena sativum*) are commonly malted for brewing purposes as well. The malting process begins with the hydration of the cereal grains by steeping them in water (temperatures usually around 12 - 18° C). The grains are then drained, spread out, and allowed to germinate in a cool and moist atmosphere. During germination, the grains produce and accumulate enzymes and sugars, which will be utilised later in the brewing process, and their starchy endosperm becomes partly degraded. After germination has progressed sufficiently, the process is halted by kilning the grains. Kilning involves passing a current of warm air through the grains, to dry and lightly cure them. Depending on the temperature and contact time of the air, different colour, flavour and enzyme activity changes of the malt are achieved. Pale malts are kilned at lower temperatures, and hence have high enzyme activity, while darker and heavily roasted malts, which are kilned at higher temperatures, don't have as much enzyme activity, but instead contribute with colour and flavour. After kilning, the malts are cooled and cleaned, after which they are ready to be used for brewing. (Briggs *et al.*, 2004)

2.1.2 Wort production

Wort production begins with the conversion of malt starches into fermentable sugars in a process called mashing. During the mashing process, the malted grains (which have usually now been milled to increase their surface area to volume ratio) and any other cereal adjuncts are steeped in warm water, at temperatures typically near the optimum temperatures of the enzymes present in the malt, until starch conversion has been achieved. The main enzymes involved in converting the amylose and amylopectin into simpler sugars are α-amylase, which cleaves α-(1,4)-links within the starch chain, and β-amylase, which cleaves the second α-(1,4)-link from the non-reducing end of starch chains. This releases a variety of simpler saccharides, including maltose, glucose, fructose, sucrose, maltotriose, and maltotetraose, as well

as dextrins, glucans, and pentosans. Mashing also involves hydrolysis of non-carbohydrate compounds, such as proteins, peptides and nucleic acids, producing amino acids, oligopeptides and nucleotides. After the mashing process, the liquid, known as wort, containing the dissolved sugars and other yeast nutrients, is separated from the solids of the mash and usually transferred to a separate vessel for boiling. (Briggs *et al.*, 2004)

The wort is then boiled together with hops or hop products, which contribute bittering and aroma compounds, for 1 - 2 hours. The hops do not only contribute to the flavour and aroma of the beer, but some of the hops compounds and derivatives (e.g. the isomerized α-acids) possess antimicrobial properties (Simpson, 1993). During the boil other changes in the wort also occur, including coagulation of proteins, killing of any microbes present, evaporation of unwanted flavour and aroma compounds, concentration of the sugars present, and colour darkening through Maillard reactions. If adjuncts (such as sugars or syrups) are to be added to the beer, they are usually added to the boil. The purposes of the adjuncts are to increase sugar concentration, as well as modify flavour, aroma and colour of the beer. After the boil the wort is cooled, and any solid matter, such as hop matter and precipitated proteins, is separated, e.g. through filtration or with a whirlpool tank. After cooling, the wort is usually oxygenated and then inoculated with yeast. (Briggs *et al.*, 2004)

2.1.3 Fermentation

Fermentation begins with the inoculation of yeast into the cooled boiled wort. The actual fermentation is usually carried out in a separate fermentation vessel, and they can be of various shapes, sizes and either closed or open to the air. The yeast used for fermentation is typically of the *Saccharomyces* family, though beer is in rare cases fermented with yeast from the *Brettanomyces* family and even bacteria as well. The species of *Saccharomyces* yeasts used for beer fermentation are typically *Saccharomyces cerevisiae* (also known as ale yeast or top-fermenting yeast) and *Saccharomyces pastorianus* (also known as lager yeast or bottom-fermenting yeast). During fermentation the yeast cells absorb and metabolize the dissolved compounds present in the wort. Other than producing new biomass, the major products of

fermentation are ethanol, carbon dioxide, and heat, which result from carbohydrate catabolism. A wide range of other products, such as esters, aldehydes, ketones, and fusel alcohols, are produced at lower concentrations, but these products can still have a large effect on the final flavour and aroma of the beer. Fermentation temperature has a large effect on the spectrum of products generated during yeast metabolism, and it is usually kept at around 15 – 20° C for ale yeast strains, and around 10 – 15° C for lager yeast strains. The primary fermentation process is usually ended when the sugar content of the wort has declined sufficiently and reached a steady-state concentration. As a result of this, yeast cells also tend to flocculate and drop out of suspension. For ale yeast strains the primary fermentation usually lasts about 3 days, while for lager yeast strains the primary fermentation can take up to a week or more. (Briggs *et al.*, 2004; Vanderhaegen *et al.*, 2003)

After the primary fermentation, the flocculated yeast is usually removed from the beer and re-used for a subsequent batch, and the beer is allowed to mature at lower temperatures (traditionally close to 0° C). This phase is also known as secondary fermentation or lagering phase. The purpose of this phase is to improve the flavour and stability of the fresh beer, e.g. by lower the concentration of unwanted fermentation by-products, such as vicinal diketones, acetaldehyde and sulphur-based compounds, and the clearing of the beer through sedimentation of any remaining yeast and trub particles. The conditioning phase is more commonly used with lagers than ales, and it can last from 3 days to several weeks. (Briggs *et al.*, 2004)

2.1.4 Down-stream processing

After conditioning, the beer typically undergoes another clearing process, as any remaining solid matter is removed by filtration or centrifugation. The bright and clear beer is then carbonated (either with carbon dioxide or through the addition of a sugar solution, which will generate carbon dioxide through fermentation in the package) and transferred to a suitable package (e.g. bottle, can, keg or bulk tank). The beer can be further stabilized through pasteurization or by sterile-filtering prior to packaging. The beer is now ready for consumption, and packaging should ensure the beer retains its clarity, flavours and carbonation until consumed. (Briggs *et al.*, 2004)

2.2 Wort composition

The post-boil wort is a complex mixture of various compounds, including carbohydrates, proteins, amino acids, lipids, inorganic ions, vitamins, isomerized α-acids, terpenes, phenols, and dissolved oxygen (Axcell, 2003; Bamforth, 2003). The malt is the source for the majority of the compounds needed for sufficient yeast growth and fermentation performance (these include the carbohydrates, nitrogen-compounds, and lipids amongst others), while adjuncts can be used to adjust proportions of individual compounds (e.g. sugars or salts) in the wort. The sugar and amino acid content of an 8.8° Plato example wort, made from 100% pilsner malt, is presented in Table 1 (Coghe *et al.*, 2005). Carbohydrates typically constitute around 90% of the dissolved compounds in wort, and of these around 70 – 80% are fermentable sugars while 20 – 30% are non-fermentable carbohydrates, such as dextrins and other oligosaccharides (Stewart and Russell, 1993). Typically, maltose and maltotriose dominate the sugar spectrum of the fermentable sugars.

The protein concentration of the wort varies depending on the quality of the malts used, the mash pH, and the mash temperatures, but is usually around 1000 – 1300 mg / l in the beginning of the mash and around 400 – 600 mg / l post-mash and pre-boil (Lewis and Serbia, 1984; Lewis and Wahnon, 1984). Boiling reduces protein content further by around 10 – 25% as a result of the hot and cold break precipitation (Brey *et al.*, 2002; Briggs *et al.*, 2004; Jin *et al.*, 2009). During the mash, proteolytic enzymes break down proteins into amino acids and shorter peptides, which remain in the wort until fermentation. The amount and type of amino acids present in the wort also depend on the malt quality (i.e. enzymes and proteins present) and mash conditions (primarily temperature and pH). When adjuncts, such as cereals, syrups and sugars, are used to partially replace the malt as the sugar source, nitrogen content in the wort usually decreases proportionally to the amount of adjunct used. In a study by Fumi *et al.* (2009), total nitrogen content and free amino nitrogen content of their wort dropped by 31% and 39% respectively, when 37.6% of the malt was replaced with maize grist.

Table 1 – The sugar and amino acid concentrations in an 8.8° Plato example wort (Coghe *et al.*, 2005).

Compound	**Concentration (mg / l)**
Sugars	
Glucose	$6.4 \cdot 10^3$
Fructose	$1.7 \cdot 10^3$
Sucrose	$4.8 \cdot 10^3$
Maltose	$38.2 \cdot 10^3$
Maltotriose	$9.9 \cdot 10^3$
Amino Acids	
Proline	340
Leucine	117
Arginine	105
Phenylalanine	103
Valine	102
Glutamine	89
Alanine	86
Tyrosine	74
Lysine	65
Isoleucine	62
Asparagine	53
Aspartic Acid	49
Serine	44
Glutamic Acid	43
Threonine	43
Tryptophan	41
Histidine	41
Glycine	27
Methionine	21

2.3 Yeast metabolism

During fermentation, a wide range of biochemical reactions occur in the wort as a result of growth of the pitched yeast cells. *Saccharomyces* spp. brewing strains are heterotrophic and facultative anaerobes. The yeast cells can utilize a wide range of organic molecules as energy sources, and their metabolism can either be aerobic and oxidative or fermentative depending on the presence of oxygen and the nature of the energy source. Yeast metabolism is complex and versatile, and depending on the genotype of the yeast strain and the growth environmental conditions, a variety of different reactions can occur and consequently a variety of different products can be formed. Brewing yeast strains are usually able to adapt and grow in various growth conditions, but to be able to produce beer of a consistent quality with acceptable fermentation performance, the desired yeast strain and suitable fermentation conditions should be utilized. (Briggs *et al.*, 2004)

Ale yeast and lager yeast strains naturally vary in fermentation performance and metabolism. Ale yeast strains, i.e. *S. cerevisiae,* have been used and available for thousands of years, lending them a much larger diversification than the lager yeast strains, i.e. those of *S. pastorianus* (previously known as *Saccharomyces carlsbergensis*, and is a hybrid between *S. cerevisiae* and *Saccharomyces eubayanus*), which are only a couple hundred years old (Caesar *et al.*, 2007; Dunn and Sherlock, 2008; Libkind *et al.*, 2011; Naumova *et al.*, 2005). Because of their genetic differences, differences in physiology between the two brewing yeast species arise as well, including different surface hydrophobicity, lower optimum growth temperature for lager strains compared to ale strains, and different sugar utilization capabilities (e.g. lager yeast strains can utilize melibiose and usually utilize maltotriose more efficiently than ale strains) (Dengis *et al.*, 1995; Giudici *et al.*, 1998; Turakainen *et al.*, 1993; Zheng *et al.*, 1994).

The preferred carbon and energy sources of *Saccharomyces* spp. are carbohydrates. Through oxidation of carbohydrates, the yeast cells can release energy, uphold the intracellular redox balance, and generate intermediates for biosynthesis. Sugar uptake into the yeast cells mainly occurs through the action of transporter proteins, and

transport can be either passive (by facilitated diffusion), as in the case of monosaccharides, or active (through proton symporters), as in the case of α-glucosides (Bisson *et al.*, 1993; Lagunas, 1993). The main carbohydrate catabolism pathway in *Saccharomyces* spp. is the Embden-Myerhof-Parnas pathway (also known as glycolysis), where glucose is converted into two molecules of pyruvate. Maltose and maltotriose are transported into the cell through specific transporters, and are then hydrolysed to glucose molecules within the cell by an α-glucosidase enzyme (Novak *et al.*, 2004). Even under aerobic conditions, fermentative metabolism is dominant in *Saccharomyces* spp., where pyruvate is first decarboxylated to acetaldehyde, which is then further reduced to ethanol by a NADH-dependent alcohol dehydrogenase. Glucose is the preferred sugar source of *Saccharomyces* spp., and its intracellular presence represses the transcription of genes encoding for enzymes that are required for the utilization of other sugars (Gancedo, 1998). This process is known as catabolite repression, and it is the reason why glucose is the first of the wort carbohydrates metabolized during fermentation. (Briggs *et al.*, 2004)

The assimilation and metabolism of wort nitrogen compounds plays 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 (e.g. yeast proteins), rather they are catabolised, and any required amino acids are synthesized from catabolic intermediates (Pierce, 1987). A minimum free amino nitrogen (FAN) content in wort of 100 ppm is usually recommended for maintaining healthy yeast and a sufficient fermentation rate, though successful fermentations of wort with a FAN content of 51 ppm have been performed (Bajomo and Young, 1994; O'Connor-Cox and Ingledew, 1989; Pierce, 1987). FAN contents above 150 ppm are mentioned by various sources for optimum fermentation performance (Enari, 1974; O'Connor-Cox and Ingledew, 1989). Insufficient FAN content in wort may lead to incomplete fermentation (i.e. low attenuation) and slow fermentation rates. In environments of excess FAN, the

fermentation rate and ethanol productivity is increased, since carbon flow through the glycolytic pathway is maximised (as no glycolytic products need to be utilised 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 (e.g. iso-butanol is formed from α-ketoisovalerate, an intermediate in the valine anabolic and catabolic pathways) (Hazelwood *et al.*, 2008; O'Connor-Cox and Ingledew, 1989). Yeast growth rate is also increased at increased concentrations of FAN in the wort (Piddocke *et al.*, 2011).

2.3.1 Amino acid uptake

The amino acids present in wort have been divided into four groups by Jones and Pierce (1964), depending on their general uptake rate in yeast (this classification does not necessarily apply to all different strains of brewing yeast). The four groups and the amino acids that belong to them are presented in Table 2 (Jones and Pierce, 1964). The Group A amino acids are characterized by fast absorption into the yeast cells (usually complete absorption within 20 hours), Group B amino acids are gradually absorbed into the yeast cell, Group C amino acids are slowly absorbed into the cell after a 'lag phase' of around 12 hours, while proline, despite being the most abundant amino acid in wort, is only marginally absorbed. Other studies (Lekkas *et al.*, 2007; Palmqvist and Äyräpää, 1969; Perpéte *et al.*, 2005) on the absorption of amino acids by brewing yeast in brewery conditions report similar classifications, with 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 (see section 2.3.2). As can be seen from Table 2, valine has a moderate uptake rate in yeast, and as shown by, e.g. Garcia *et al.* (1994) and Perpéte *et al.* (2005), valine uptake is usually slow during the first approximately 12 hours of fermentation, during which the majority of the Group A amino acids are absorbed into the cell. Romkes and Lewis (1971) also observed in their study on the amino acid uptake rate by *Saccharomyces carlsbergensis* (synonymous with *S. pastorianus*)

harvested from a stationary growth phase, that valine was among the group of amino acids with longest lag phases before absorption began. Kielland-Brandt *et al.* (1990) also observed that valine uptake was poorer in *S. carlsbergensis* than in *S. cerevisiae*, suggesting that valine could be classified as a Group C amino acid for *S. pastorianus* fermentations (as suggested by Palmqvist and Äyräpää, (1969)).

Table 2 – The classification of wort amino acids based on their uptake rate in *Saccharomyces cerevisiae* (Jones and Pierce, 1964).

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

There are various transporter 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) (de Boer *et al.*, 1998; Didion *et al.*, 1998; Kodama *et al.*, 2001a). The transport of amino acids into *S. cerevisiae* is active, and the amino acid permeases are driven by proton symport (Seaston *et al.*, 1973). The transporters aren't 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, such as valine, and it is down-regulated by the presence of preferred amino acids, such as glutamine and aspargine, and ammonia, suggesting it is only responsible for transporting a minor fraction of the valine in wort during fermentation (Grenson *et al.*, 1970; Stanbrough

and Magasanik, 1995). Kodama *et al.* (2001b) 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 confirms that there are several transporters that transport valine into the cell during fermentation.

Kodama *et al.* (2001a) studied the transcription of a Bap2p-encoding gene (Lg-*BAP2*, identical to Bap2p-encoding gene in *Saccharomyces bayanus*) in a lager yeast strain, and found that it was repressed during the beginning of the fermentation and that transcription was induced when the majority of the amino acids in the wort were absorbed. Similar results were observed by Gibson *et al.* (2009) 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.* (2001a) was not able to distinguish any certain compound or mechanism that induced transcription, but he 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 (Didion *et al.*, 1996). Schoondermark-Stolk *et al.* (2006) 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 by the pH drop of the beer occurring during fermentation, causing an increase in valine uptake as fermentation progresses. Verbelen *et al.* (2009b) studied the effect of pre-oxygenating a lager yeast and the wort before pitching on the expression of various genes, including *BAP2*, during fermentation and concluded that the initial expression level (measured at 1 hour after pitching) of *BAP2* was higher for a pre-oxygenated yeast and aerated wort compared to a non-pre-oxygenated yeast and aerated wort, but after 4.5 hours 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.

2.3.2 Diacetyl production

Diacetyl, or 2,3-butanedione, and 2,3-pentanedione are vicinal diketones (VDK) that are formed during beer fermentation as by-products of amino acid synthesis (valine and isoleucine respectively) in *Saccharomyces* spp. yeast, and they can have a significant effect on the flavour and aroma of beer. Diacetyl is known for its butter-like and butterscotch-like flavour, and its flavour threshold is usually reported as around 0.1 - 0.2 ppm in lager and 0.1 – 0.4 ppm in ales (Meilgaard, 1975; Wainwright, 1973), although flavour thresholds as low as 17 ppb (Saison *et al.*, 2009) and 14 – 61 ppb (Kluba *et al.*, 1993) have been reported. 2,3-pentanedione has a similar flavour to diacetyl, though often described as more toffee-like, but it has a higher flavour threshold of around 0.9 – 1.0 ppm (Meilgaard, 1975; Wainwright, 1973). VDKs are more easily detectable in lighter beers, where the flavour is not masked by malt and hop flavours. Presence of VDKs above their flavour threshold in beer is generally regarded as a defect, since their flavour is undesirable in most beer styles and it can also indicate microbial contamination by *Lactobacillus* spp. or *Pediococcus* spp. (Boulton and Quain, 2001). Nevertheless, diacetyl in detectable (yet low) amounts is acceptable in some beer styles, such as Bohemian Pilsner and some English Ales (BJCP, 2008).

The generally accepted pathways for diacetyl and 2,3-pentanedione formation and reduction in *Saccharomyces* spp. are presented in Figure 2 (Chuang and Collins, 1968; Radhakrishnan and Snell, 1960; Strassman *et al.*, 1958; Suomalainen and Ronkainen, 1968). 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. In the valine biosynthesis pathway, the reaction between α-acetolactate and 2,3-dihydro-isovalerate is rate-limiting, and thus during fermentation and yeast growth, some α-acetolactate transfers out through the cell membrane into the wort (Dillemans *et al.*, 1987). In the wort, 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 rate of spontaneous oxidative decarboxylation is

increased at higher temperatures, in the presence of oxygen (the reaction can occur under anaerobic conditions as well, where Cu^{2+}, Fe^{3+} and Al^{3+} ions function as electron acceptors), and at acidic pH values (at extreme acidic conditions the non-oxidative decarboxylation to acetoin is favoured though) (Wainwright, 1973). The non-oxidative decarboxylation into acetoin can be encouraged by heating under anaerobic conditions and by maintaining a low redox potential in the wort (Kobayashi *et al.*, 2005a). 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.

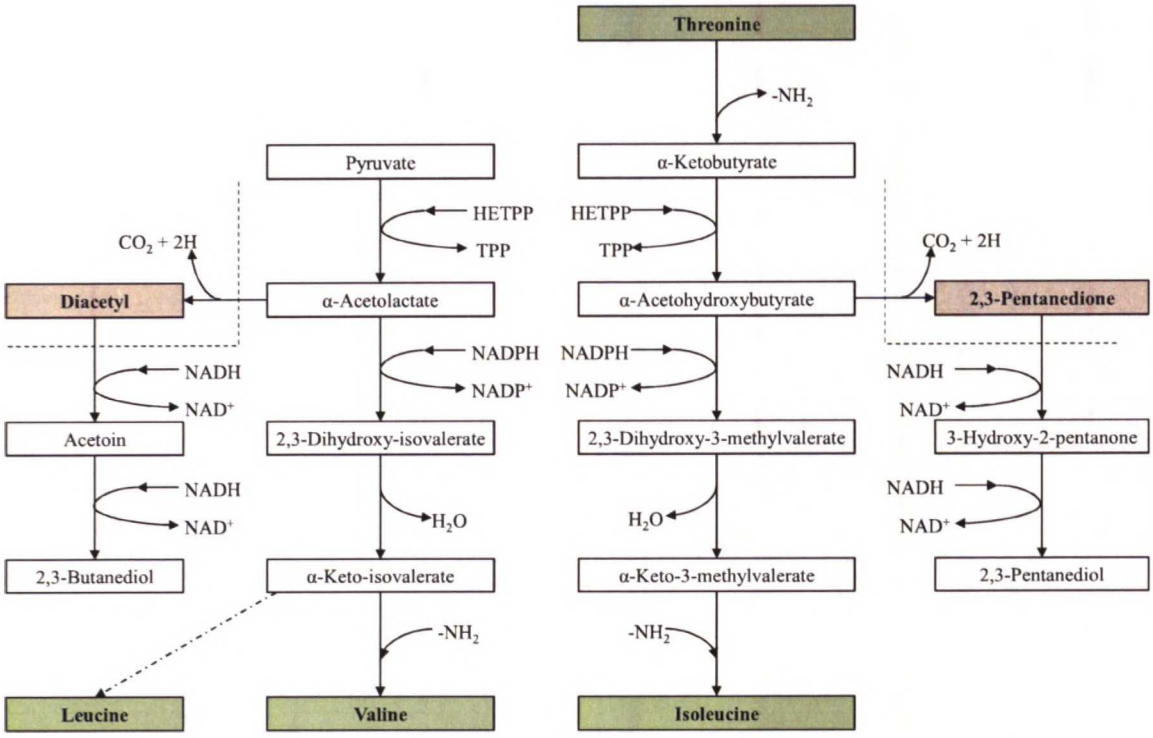

Figure 2 – The pathways for diacetyl and 2,3-pentandione formation and reduction, as well as valine and isoleucine synthesis, in *Saccharomyces* spp. yeast. The dotted line depicts the cell boundary. The dashed arrow from α-keto-isovalerate to leucine depicts a series of six reactions. The boxes featuring red backgrounds are vicinal diketones and the boxes with green backgrounds are amino acids. (Chuang and Collins, 1968; Radhakrishnan and Snell, 1960; Strassman *et al.*, 1958; Suomalainen and Ronkainen, 1968)

2.3.3 Diacetyl control

The yeast is also capable of assimilating and reducing the formed VDK into diols, which have much higher flavour thresholds compared to 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 (Meilgaard, 1975; Hughes *et al.*, 2001). 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 (Bamforth and Kanauchi, 2004). In a study on ale and lager yeast strains, Murphy *et al.* (1996) observed a specific acetoin reductase enzyme in the lager strains, which was not present in the ale strains, which instead expressed a dehydrogenase enzyme active on both diacetyl and acetoin. Bamforth and Kanauchi (2004) also observed a larger variety of diacetyl reductase enzymes in lager yeast compared to ale yeast. The actual reduction of diacetyl in the yeast is rapid, as shown by Boulton and Box (2003), where external additions of diacetyl to active fermentations resulted in diacetyl peaks rapidly declining back to the diacetyl levels of the control fermentation. Hence it can be concluded that the reduction of diacetyl in the yeast is not rate-limiting in the pathway. However, in the same study, the width of the diacetyl peak after diacetyl addition increased the later during fermentation the addition was added, suggesting that yeast physiological condition does have some influence on the rate of diacetyl reduction.

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 (Garcia *et al.*, 1994). 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 (Garcia *et al.*, 1994; Saerens *et al.*, 2008b; Wainwright, 1973). Bamforth and Kanauchi (2004) 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. those closer to 3.5). Several sources (Garcia *et al.*, 1994; Kobayashi *et al.*, 2005a; Rondags *et al.*, 1996) 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 varies from around 3.7 to 5.0 depending on style and ingredients (Briggs *et al.*, 2004).

Since diacetyl is directly linked to the valine biosynthesis pathway, the amount of valine present in the wort affects the amount of diacetyl generated during fermentation, as it has been shown that valine strongly inhibits the acetohydroxyacid synthase enzyme, responsible for catalysing the formation of α-acetolactate from pyruvate (see Figure 1) (Magee and de Robichon-Szulmajster, 1968). Hence, the more valine present in the wort, the less α-acetolactate will be synthesized, as the catalysing enzyme is inhibited, and consequently less diacetyl will be formed as well. Nakatani *et al.* (1984) studied the effect of external 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 on wort with various original gravities, free amino nitrogen and valine content, Petersen *et al.* (2004) 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), while high concentrations of valine in the wort resulted in single-peak diacetyl profiles with a lower maximum diacetyl level compared to 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 on the relationship between wort valine concentration and diacetyl concentration can be drawn. Cyr *et al.* (2007) 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. Hence it can be concluded that there is a direct relationship between valine uptake (thus also wort valine concentration) and diacetyl production.

In the same study by Cyr *et al.* (2007), it was observed that the maximum valine uptake for yeast cells pitched dry was up to 6 times lower than that of yeast cells pitched from a fresh slurry, consequently causing a significantly higher diacetyl peak, indicating that the yeast drying process can invoke changes in the cells membranes, influencing valine transport into the cell. The rate of valine transport into the 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, valine must instead be biosynthesized, 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 would also increase the amount of 2,3-pentanedione produced. Romkes and Lewis (1971) 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 glucose solution with aeration. These results also agree with those by Verbelen *et al.* (2009b) on increased *BAP2* expression as a result of pre-oxygenating the yeast before pitching. Valine addition to the pre-treatment solution and adjusting the pH of the pre-treatment to below 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 (Schoondermark-Stolk *et al.*, 2006). Kodama *et al.* (2001a) on the other hand reported decreased expression of the *S. bayanus*-derived *BAP2* gene and no change

of expression of the *S. cerevisiae*-derived *BAP2* gene in a lager yeast strain in an YPM medium with 1 mM sorbate (pH 4.5) compared to the control YPM medium, suggesting that acidic conditions could have a negative effect on the production of branched-chain amino acid permeases.

The general free amino nitrogen (FAN) content (and especially the concentration of Group A amino acids) of the wort may also affect the valine uptake rate and consequently diacetyl production. Pugh *et al.* (1997) observed during trials with lager yeast fermentations of wort with varying FAN content, 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. This can be explained by the fact that Group A amino acids are absorbed into the yeast cells first, while the Group B amino acids are absorbed slowly during this so called lag phase. As the FAN content increases, while the amino acid profile stays constant, the absolute concentration of Group A amino acids increases, extending their absorption phase and the lag phase of the absorption of Group B amino acids. Therefore the yeast cells need to synthesize the necessary valine (and isoleucine) for growth until any preferred amino acids are used up and valine is absorbed, thereby affecting the production of VDK. The results indicate that diacetyl production during fermentation could be minimized by lowering the general FAN content of the wort to levels sufficient for proper yeast growth, and then supplying external valine to increase the valine wort concentration. Verbelen (2009) reports a lower diacetyl production rate and a simultaneous increased valine uptake rate and *BAP2* expression level in lager yeast for fermentations of 18° Plato worts containing adjuncts (FAN contents of around 150-210 ppm) compared to 18° Plato all-malt wort (FAN content around 300 ppm). These results also confirm that valine uptake rate is increased and diacetyl production is decreased as the concentration of rapidly absorbable amino acids in the wort is decreased.

Pitching rate and cell density also affects the amount of diacetyl formed and present in beer at the end of fermentation, as it has been observed that the concentration of

diacetyl in beer at the end of fermentation increases with an increased pitching rate. In trials with various lager yeast strains, Verbelen *et al.* (2008) observed over ten-fold increases in diacetyl concentrations post-fermentation for the majority of the studied yeast strains when pitching rate was increased from 20×10^6 viable cells / ml to 80×10^6 viable cells / ml. Verbelen *et al.* (2009a) also observed increasing concentrations of diacetyl post-fermentation in beers fermented with an industrial lager yeast strain as pitching rate was increased step-wise from 10×10^6 viable cells / ml to 120×10^6 viable cells / ml, with a maximum diacetyl concentration in the beer pitched with 80×10^6 viable cells / ml. This can be explained by the fact that 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. On the other hand, Erten *et al.* (2007) 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.* (2008) and (2009a) lasted for approximately 2 to 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.* (2013) also observed increased concentrations of VDK in beer fermented for 72 hours with an osmotolerant strain, compared to the beer fermented for 193 hours with the control strain.

Other strategies for diacetyl control in beer include reducing the activity of, or disrupting the genes coding for, the acetohydroxyacid synthase (AHAS) enzyme, responsible for catalysing the formation of α-acetolactate from pyruvate (see Figure 1), increasing the metabolic flux through the pathway from α-acetolactate to valine, exposing the fermenting wort to, or introducing into the yeast the genes coding for, the α-acetolactate decarboxylase enzyme, which catalyses the non-oxidative decarboxylation of α-acetolactate into acetoin, or passing the fermented beer through

immobilized yeast cell reactors. Wang *et al.* (2008) report that diacetyl production was decreased with 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 to fermentations with the unmodified strains. The disruption caused lower expression of AHAS and consequently less diacetyl was formed. The use of genetically engineered yeast strains in commercial beer has not yet been possible though due to lack of consumer acceptance and legal regulations (Saerens *et al.*, 2010). Barton and Slaughter (1992) 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.

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* (also known as *YCL009c*) gene. Cullin *et al.* (1996) observed that the inhibiting effect of valine on AHAS activity in *S. cerevisiae* was lost with the deletion of *YCL009c*. Similar results were noticed by Pang and Duggleby (1999) 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 to 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 to when both *ILV2* and *ILV6* were expressed, suggesting that the *ILV6*-encoded subunit not only is responsible for feedback inhibition, but it also enhances the general activity of AHAS. Cullin *et al.* (1996) on the other hand, did not observe any decrease of AHAS activity with the deletion of *YCL009c*, but Pang and Duggleby (1999) 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.* (2011) found lower expression levels of *ILV6* during fermentation in a lager yeast strain with low diacetyl production compared to 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 to 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 Figure 2). This can be achieved by increasing the activities and expression of the enzymes catalysing the reactions in the pathway. The acetohydroxyacid reductoisomerase enzyme, coded by the *ILV5* gene, is responsible for the conversion of α-acetolactate to 2,3-dihydroxy-isovalerate in *Saccharomyces* yeasts (Petersen and Holmberg, 1986). Dillemans *et al.* (1987) examined the effect of introducing an *ILV5* gene coding for a highly active acetohydroxyacid reductoisomerase enzyme from a mutant *S. cerevisiae* strain into a 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 to 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 (1995), with the introduction of *ILV5* genes in plasmids to lager yeast, resulting in the expression of more acetohydroxyacid reductoisomerase and less diacetyl formation. Kusunoki and Ogata (2012) also successfully integrated copies of *ILV5* genes upstream of the *S. cerevisiae*-type *ILV2* gene in a *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 quicker (67 hours compared to 160 hours) compared to the control strain. Lu *et al.* (2012) also observed less diacetyl production throughout the fermentation with a lager yeast strain modified for *ILV5* overexpression compared to the unmodified strain. Bussey and Umbarger (1969) 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 (1973) showed that threonine deaminase, responsible for catalysing the conversion of threonine to α-ketobutyrate in the first step of the isoleucine biosynthesis pathway (see Figure 2), 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 Figure 2), is NADPH-dependent (Petersen and Holmberg, 1986). Increased flux towards valine, resulting in less α-acetolactate leaking 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.* (2012) report an increased yield of L-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.* (2010) 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 fermentation rate of the yeast and flavour profile of the beer could be affected by metabolic changes caused by changes in the cofactor balance.

Due 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 (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 (Godtfredsen and Ottesen, 1982; Godtfredsen *et al.*, 1984) and by introducing the gene encoding for the enzyme to brewer's yeast (Blomqvist *et al.*, 1991; Kronlöf and Linko, 1992; Shimizu *et al.*, 1989; Sone *et al.*, 1988). 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. The problem regarding the use of genetically engineered yeast strains and the use of bacterial DNA comes up again, and these methods are therefore not used commercially.

The reduction of diacetyl in beer that has finished fermentation can be sped up with the use of immobilized yeast cells (Mensour *et al.*, 1997; Verbelen *et al.*, 2006). 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) unit, VDK concentrations and maturation times can be rapidly decreased. This reduction method, like the control of the wort amino acid profile, requires no use of genetically engineered yeast, and it is thus in commercial use. Then again, the use of an immobilized yeast cell reactor requires a significant investment by the brewery.

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 (Jones, 2005). By mashing in conditions favouring the activity of those enzymes releasing more valine into the wort, the valine content of the wort could potentially

be increased, which in turn could lead to decreased diacetyl formation during fermentation. Schwarz *et al.* (2012) investigated the effect of various 'mashing-in' (i.e. the 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, pH of 5.4 and time of 60-75 minutes 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 (2009) also reports that 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.

Table 3 – The amino acid content (mg per 100 g of malt) of worts prepared with various malts (Samaras *et al.*, 2005).

Amino Acid	**Green malt**	**Lager malt**	**Pale malt**	**Cara malt**	**Crystal malt**	**Black malt**	**Chocolate malt**
Valine	6.69	7.35	3.14	2.67	1.57	0.51	0.51
Leucine	8.03	8.39	4.19	1.6	0.52	0.51	0.51
Isoleucine	4.01	4.2	2.1	0.53	0.52	0.51	0.51
Total	111.04	146.9	61.84	38.91	17.29	6.18	4.12

The type of malts 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 (Jones, 2005). Samaras *et al.* (2005) studied the effect of using various malt types on the amino acid content of the wort (see Table 3), 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 qualities of protein. The valine and crude protein content of various cereal grains is presented in Table 4. Oat protein has 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.* (2011) showed that brewing a 100% oat malt beer is possible, but extract yield was decreased and wort viscosity increased compared to wort produced from barley malt. Also, diacetyl concentration at the end of fermentation was higher for the beer brewed from oat malt, compared to the one brewed from barley malt.

Table 4 – The valine and crude protein content of various cereal grains.

Cereal grain	**Valine content (% of total protein)**	**Crude protein (% of total grain)**	**Source**
Barley	4.7	14.3	Morey, 1983
Barley	4.9	15.4	McElroy *et al.*, 1949
Barley	4.9	-	Shewry, 2007
Oats	5.4	15.1	Morey, 1983
Oats	5.7	14.3	McElroy *et al.*, 1949
Oats	5.0	14.3	Wu *et al.*, 1972
Oats	5.3	-	Shewry, 2007
Rye	4.4	15.5	Morey, 1983
Rye	4.8	-	Shewry, 2007
Wheat	4.4	16.4	Morey, 1983
Wheat	4.4	18.9	McElroy *et al.*, 1949
Wheat	4.4	-	Shewry, 2007

Valine supplementation to wort or modification of wort amino acid profile can also potentially result in unwanted side-effects, such as an increased production and concentration of higher alcohols, valine itself contributing to the flavour of the beer, high levels of residual amino acids acting as nitrogen sources for potential spoilage microbes, decreased yeast growth and increased costs. In *Saccharomyces* spp., higher alcohols (or fusel alcohols) are formed through the catabolism of certain amino acids (e.g. branched-chain amino acids) via the Ehrlich pathway (Hazelwood *et al.*, 2008). Valine is converted into isobutanol (2-methylpropanol) through a transamination, decarboxylation and oxidation reaction. Isobutanol has a flavour threshold of around 200 ppm in beer and it has an alcoholic and solvent-like flavour (Meilgaard, 1975).

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 (Haraguchi *et al.*, 2011; Mukai *et al.*, 2007) and it has a bittersweet flavour (Schiffman *et al.*, 1979). 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 during fermentation, but the rest remains in the wort and may cause off-flavours. High concentrations of amino acids in the fermented beer may also affect visual quality of the beer as well (see Section 2.4.1). Amino acids may also undergo Strecker degradation into aldehydes during beer aging when oxygen is present, suggesting that the long-term stability of amino acid supplemented beer may be lessened (Vanderhaegen *et al.*, 2006). In previous sections it has been mentioned that the FAN content of the wort affects yeast growth during fermentation and supplementation of valine may affect the uptake rate of other amino acids. Hence, over-supplementation of valine may cause decreased yeast growth, as a result of modified amino acid uptake rate patterns, which in turn can affect vital beer properties such as the alcohol content and yeast attenuation. Finally, the costs of valine supplementation to wort must be less than the costs saved from a decreased maturation time if the technique is to be profitable for a brewery.

2.3.3.1 Summary of methods to control diacetyl production during fermentation

The various methods for decreasing the amount of diacetyl produced during wort fermentation described in the previous section 2.3.3 are presented in Table 5. 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 formed α-acetolactate or diacetyl.

Table 5 – Methods for decreasing the amount of diacetyl produced during the fermentation of beer.

Method	GMO	Mechanism	Disadvantages
Increased conditioning time [1]		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 [2]		Increased reaction rate for the oxidative decarboxylation of α-acetolactate to diacetyl.	pH affects the flavour and mouthfeel of beer.
Valine supplementation [3]		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 [4]		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* [5]	X	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* [6]	X	Increases the flux through the valine biosynthesis pathway, resulting in less α-acetolactate leaking out of the cell.	GMO. Potential increase in higher alcohols and esters.
Introduction and expression of ALDC-encoding gene [7]	X	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 is available.
Supplementing ALDC enzyme [8]		See above.	Costly if added in batches, and requires considerable investment if encapsulated enzymes are used.
Using immobilized yeast cells [9]		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.

Table 5 (cont.) – Methods for decreasing the amount of diacetyl produced during the fermentation of beer.

Method	GMO	Mechanism	Disadvantages
Optimizing mash conditions [10]		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 [11]		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 wort after the mash. See mechanism for 'Valine supplementation'.	Activity of the various cytolytic, proteolytic and amylolytic enzymes in the malt may be affected, resulting in decreased extract yields and modified wort properties.
Quality and type of malt [12]		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.

[1] (Bamforth and Kanauchi, 2004), [2] (Bamforth and Kanauchi, 2004; Garcia *et al.*, 1994; Kobayashi *et al.*, 2005a; Rondags *et al.*, 1996), [3] (Cyr *et al.*, 2007; Magee and de Robichon-Szulmajster, 1968; Petersen *et al.*, 2004; Schoondermark-Stolk *et al.*, 2006), [4] (Pugh *et al.*, 1997; Verbelen, 2009), [5] (Duong *et al.*, 2011; Kusunoki and Ogata, 2012; Wang *et al.*, 2008), [6] (Dillemans *et al.*, 1987; Kusunoki and Ogata, 2012; Lu *et al.*, 2012; Mithieux and Weiss, 1995), [7] (Blomqvist *et al.*, 1991; Kronlöf and Linko, 1992; Shimizu *et al.*, 1989; Sone *et al.*, 1988), [8] (Godtfredsen and Ottesen, 1982; Godtfredsen *et al.*, 1984), [9] (Mensour *et al.*, 1997; Verbelen *et al.*, 2006), [10] (Gómez Guerrero, 2009; Jones, 2005; Schwarz *et al.*, 2012), [11] (Jones, 2005; Samaras *et al.*, 2005), [12] (Klose *et al.*, 2011; McElroy *et al.*, 1949; Morey, 1983; Shewry, 2007; Wu *et al.*, 1972)

2.4 Beer quality

Since beer is a complex product meant for human consumption, it is important for the brewers to maintain high quality products to ensure customer satisfaction and safety. 'Quality' can be defined in a variety of ways and is also subjective, but generally in regards to beer, a quality product can be seen as one that meets both the brewer's and the customer's specifications, is produced with consistent attributes, has a long shelf-life, and is safe to consume. To maintain a high quality of their products, breweries practise quality control and assurance, which can involve e.g. implementation of ISO standards, HACCP management, regular analysis of raw materials and products by standard methods, product and process development, consumer surveys, and market analysis. Technical quality attributes related to beer include visual appearance, taste and aroma, colloidal stability, microbiological stability and even nutritional aspects, and these attributes can be assessed in a variety of ways (Bamforth, 2008).

2.4.1 Visual quality

The visual quality of beer involves three major attributes: foam head, clarity and colour. These attributes are of importance since they affect the consumer before they even have consumed the product. Beer foam is a colloidal system involving a gaseous phase dispersed in a liquid or solid phase, and the foam does not only provide visual appeal to the beer, but also functions as a gas exchange surface, from which beer aroma compounds are released to the consumer, and provides mouthfeel. The features describing good foam quality are both dependent on beer style and personal preferences, but generally include stability (i.e. how long will the foam maintain), quantity (i.e. how much foam is formed), lacing (i.e. does the foam cling to the glass as it collapses), and creaminess (i.e. the size of the bubbles in the foam) (Evans and Bamforth, 2008; Hughes *et al.*, 2001).

Beer foam is formed as a result of a combination of various events: bubble formation, drainage, creaming, coalescence and disproportionation. Bubbles are

formed as a result of nucleation of the supersaturated carbon dioxide in beer, stimulated by the presence of nucleation sites (e.g. particles and scratches) and dispense mode. The size of the bubbles relates to the size of the nucleation site, and smaller bubbles are usually more desirable for higher quality foam. As foam is formed, through the rise of the formed bubbles to the surface, it is initially 'wet', i.e. the continuous phase of the colloidal system contains much liquid. Much of the liquid drains from the foam as a result of gravity, in a process called drainage, leaving the foam 'dry', i.e. the continuous phase consists mostly of a solid network of bubble walls. Drainage of the foam reduces its stability as a result of the weakening of bubble film (Evans and Bamforth, 2008; Hughes *et al.*, 2001).

Creaming is defined as the rising of bubbles through the beer and the addition of these bubbles into the foam. The amount of creaming depends on the surface tension, density and carbon dioxide content of the beer. Coalescence is the merger of two bubbles, as a result of the rupturing of the bubble film between them. This newly formed bubble is larger and less stable than its precursors, and the overall foam stability is reduced as well. Hydrophobic particles (e.g. from an unclean glass) can usually initiate coalescence. Disproportionation is another form of bubble fusion, and it results from gas diffusion between bubbles. The carbon dioxide in smaller bubbles diffuses into larger bubbles, with a lower Laplace pressure, causing the growth of larger bubbles and the disappearance of smaller ones. The rate of disproportionation depends on a variety of different factors, including bubble film thickness, surface tension, and pressure (Evans and Bamforth, 2008).

Beer contains a range of different compounds that affect, both positively and negatively, foam stability and head retention. Compounds that have shown to be beneficial for foam stability include proteins, hop acids, polyphenols, non-starch polysaccharides, divalent metal cations and ethanol at low concentrations. Lipids, high ethanol concentrations, basic amino acids, higher alcohols and esters negatively affect foam stability on the other hand. The major proteins involved in foam formation are lipid transfer proteins (LTP1), protein Z and hordeins, but up to 30 different proteins have been found in beer and beer foam (Hao *et al.*, 2006). The

amount of foam proteins in beer is affected by a variety of factors, including the malt protein content, as higher malt protein content results in a higher protein Z concentration in the beer, malt treatment, as higher temperatures and longer kilning times will result in less extractable foam proteins, mash conditions, as proteolysis affects the amount of hordeins formed, and the use of cereal adjuncts, as certain cereal adjuncts, such as wheat and rye, have shown to increase foam stability and quality, while others, such as rice and maize, have shown to decrease foam stability and quality (Evans and Hejgaard, 1999; Sheehan, 1997). Wort boiling, proteinase A excretion from the yeast during fermentation and presence of hydrophobins, derived from *Fusarium* fungi infection of the malt, will also have an effect on the foam stability and quality of the beer (Sarlin *et al.*, 2005). Dilution of the wort with low-protein carbohydrate adjuncts (e.g. sugar solutions), will also dilute the concentration of foam proteins, and simultaneously indirectly reduce the amount of LTP1 during fermentation through an increased activity of proteinase A at lower FAN levels (Hao *et al.*, 2010) (Evans and Bamforth, 2008; Hughes *et al.*, 2001).

Haze in beer can be divided into two types, biological and non-biological haze. Biological haze is caused by suspended brewer's yeast or contamination by and growth of some other micro-organism. Non-biological haze is usually a more common problem, and it mainly involves protein-polyphenol interactions, but can also be caused by β-glucans, α-glucans, pentosans and oxalates. Non-biological haze can be further divided into permanent haze, i.e. haze that is present irrespective of any storage conditions, and chill haze, i.e. haze that is reversibly formed during the storage of the beer at cold conditions, but is dissolved as temperature is raised again. Many of the proteins involved in foam formation are also involved in haze formation, and proteins rich in proline (i.e. prolamins, such as hordeins) are especially susceptible for interactions with polyphenols, since their unfolded structures allow access of polyphenols and they are usually able to survive wort boiling, as well as hot and cold break extraction. Beer polyphenols originate from either the barley or the hops, and they are usually present in concentrations of 100 to 300 ppm. Only a fraction of the polyphenols present (mainly the proanthocyanidins) is involved in haze formation reactions (Asano *et al.*, 1984). These reactions involve

the formation of a soluble protein-polyphenol complex that grows up to colloidal size (when it begins scattering light) through further interactions. The amount of beer haze can be reduced by storing the beer cold for longer time periods, using kettle finings (which interact with proteins or polyphenols), centrifuging the beer, or filtering the beer (Briggs *et al.*, 2004; Leiper and Miedl, 2008).

The colour of beer is mostly dependent on the type of malt used, with heavily roasted malts contributing to a darker beer colour compared to malts kilned at lower temperatures. The malting process results in the formation of pigmented substances in the malt (as barley grains contain low amount of pigments), through Maillard reactions, caramelization and pyrolysis, while similar reactions occur during wort boiling as well. The Maillard reactions, i.e. reactions between an amino acid and a reducing sugar, result in the formation of melanoidins, which have colours ranging from yellow via red to brown depending on how long the reactions proceed (Nursten, 2005). Longer boiling times thus lead to a darker wort and beer. At pH 5.2, i.e. around that of pre-boil wort, Maillard reactions between valine and glucose result in the formation of brown-coloured products with a caramel-like, biscuit-like, malty, chocolate-like and bitter aroma (Wong *et al.*, 2008). Hence, considerable supplementation of valine to pre-boil wort could slightly affect the flavour and colour of the resulting beer. Beer colour can also be adjusted post-fermentation by adding intensely-coloured concentrated malt extracts or caramel syrups (Shellhammer, 2008).

2.4.2 Flavour quality

As with visual quality, the types and intensity of flavours that are acceptable in beer are highly dependent on style and personal preferences. Beer contains a multitude of different compounds that affect flavour, aroma and mouthfeel. These either originate directly from the raw materials (e.g. linalool from hops and furaneol from caramel malts) or are produced at some time during the production process (e.g. isohumulone during boiling and ethanol during fermentation). Since the flavour compounds have a wide range of flavour thresholds, ranging from below 1 µg/l (e.g. trans-2-nonenal) to above 10 g/l (e.g. ethanol), and removing individual components from the beer is

difficult, it is vital that the whole production process is carefully controlled to ensure consistent products. Beer maturation or conditioning is used as a method for reducing the amount of unwanted flavour compounds and improving flavour stability, but it is time-consuming and can take several weeks (Hughes, 2008).

The main groups of flavour compounds, other than the vicinal diketones (see section 2.3.2), that are removed or are decreased in concentration during the maturation phase are aldehydes (mainly acetaldehyde), sulphur compounds (mainly hydrogen sulphide and dimethyl sulphide) and volatile fatty acids. Acetaldehyde, known for a green apple-like flavour, is an intermediate of the fermentation pathway, and concentrations of acetaldehyde in the beer are usually highest in early stages of fermentation, as a result of excessive oxidation of ethanol and decarboxylation of pyruvate. After the primary fermentation is over, the yeast is able to reduce acetaldehyde back to ethanol and levels under its flavour threshold through the action of alcohol dehydrogenase enzymes. The flavour threshold of acetaldehyde in beer is usually reported as 10 ppm (Meilgaard, 1982), but thresholds as low as 1.1 ppm have been observed (Saison *et al.*, 2009). Sulphur compounds are usually present in low concentrations at the end of fermentation, but because of their low flavour thresholds, these can still have a large impact on beer flavour. Hydrogen sulphide, known for a rotten egg-like flavour, is formed and assimilated during fermentation as a result of the biosynthesis of sulphur-containing amino acids. The flavour threshold of hydrogen sulphide in beer is just 4 ppb (Meilgaard, 1982), and hence control of its concentration is important, especially in lightly flavoured beers. The concentration of hydrogen sulphide in the beer is decreased during later stages of fermentation and during conditioning as a result of assimilation by yeast cells (Oka *et al.*, 2008) (Briggs *et al.*, 2004; Hughes, 2008).

Esters play a very important role in the flavour and aroma profile of beer, and these are formed by the yeast during fermentation through intracellular condensation reactions between alcohols and acyl-CoA, catalysed mainly by alcohol acetyltransferases (such as the enzymes encoded by *ATF1*, Lg-*ATF1*, and *ATF2*). The majority of flavour-active esters in beer are lipid soluble, meaning they can leak

out of the cells into the wort, but transfer rate of the esters across the cell membrane is decreased with increasing fatty acid chain length. Esters are known for their fruit- and solvent-like flavours, and are hence both more acceptable and common in ales than in lagers. The most abundant ester in beer is ethyl acetate, formed from ethanol and acetyl-CoA, and it has a solvent-like flavour and a flavour threshold of around 20-40 ppm (Meilgaard, 1982). Other important esters in beer are isoamyl acetate (banana-like and fruity aroma), ethyl caproate (apple-like and fruity aroma), ethyl caprylate (apple-like and fruity aroma), phenyl ethyl acetate (floral aroma) and ethyl caprate (apple- and solvent-like aroma). The rate of ester synthesis during fermentation is strongly linked to lipid synthesis, as maximum rates of ester synthesis are usually observed in the latter half of fermentation when lipid synthesis is concluded and an overabundance of acetyl-CoA exists (Thurston *et al.*, 1982). Yoshioka and Hashimoto (1984) also observed that alcohol acetyltransferase activity was inhibited by unsaturated fatty acids. The rate of ester synthesis is thus dependent on the concentration of alcohols (ethanol and other higher alcohols) and acyl-CoA in the cell, and the activity of the enzymes responsible for catalysing the condensation reaction between them. These in turn are affected by various fermentation parameters such as yeast strain, original gravity, FAN content, oxygen and lipid concentration of wort, fermentation temperature, pitching rate, fermentation pressure and even fermenter design (Briggs *et al.*, 2004; Verstrepen *et al.*, 2003).

An increase in fermentation temperature (in the range of 10 to 25° C) generally results in increased concentrations of esters and fusel alcohols in the beer (Barker *et al.*, 1992; Bolat *et al.*, 2011; Sablayrolles and Ball, 1995; Saerens *et al.*, 2008a; Titica *et al.*, 2000), but exceptions in this pattern have been observed for certain esters, such as ethyl hexanoate (Saerens *et al.*, 2008a). Relationship between fermentation temperature and ester production is also strain-related, suggested to be caused by differences in alcohol acetyltransferase activities and expression, and is affected by various other fermentation parameters such as pressure and temperature profile (Nykänen and Nykänen, 1977; Ramos-Jeunehomme *et al.*, 1991). The effect of increased ester synthesis by fermentation temperature may be caused by a variety of phenomenon, including more efficient release of esters through the cell membrane

and increased yeast autolysis, increased expression and activity of alcohol acetyltransferases, increased synthesis of higher alcohols, and increased yeast growth at higher temperatures (Calderbank and Hammond, 1994; Haukeli and Lie, 1971; Mason and Dufour, 2000; Saerens *et al.*, 2008b; Suomalainen, 1981). The synthesis of higher alcohols in *Saccharomyces* spp. is strongly linked to the expression and activity of branched-chain amino acid aminotransferases (such as the enzymes encoded by *BAT1* and *BAT2*), and positive correlations between temperature, *BAT1* expression and higher alcohol production have been observed (Saerens *et al.*, 2008b; Yoshimoto *et al.*, 2002). Higher temperatures also increase expression of the branched-chain amino acid permeases encoded by *BAP2* and *BAP3*, resulting in an increased uptake of branched-chain amino acids, i.e. the precursors to higher alcohols synthesized through the Ehrlich pathway (Abe and Minegishi, 2008).

The free amino nitrogen content of the wort also affects the amount of esters and higher alcohols produced during fermentation, but differing relations have been observed. Lei *et al.* (2012) observed increased concentrations of esters and higher alcohols in beer fermented from wort with lower FAN contents, even though expression levels of *BAT1* were higher in the worts with higher FAN content. The results suggest that lower FAN contents of the wort promoted intracellular biosynthesis of amino acids, which were then transaminated into higher alcohols. Similar results were observed by Szlavko (1974), with increased concentrations of higher alcohols in beer fermented from wort with low FAN content, but differences existed between lager and ale yeast. Vidal *et al.* (2013) also report similar results, with lower higher alcohol production, but higher acetate and acyl ester concentrations, in cachaça fermented from sugar cane juice supplemented with ammonium sulphate. Kodama (2001b) on the other hand observed increased production of higher alcohols in wort supplemented with leucine, isoleucine and valine, and also increased isoamyl concentrations in beer fermented with a lager yeast strain constitutively expressing *BAP2* compared to a control strain, suggesting it is the concentrations and uptake rates of the branched-chain amino acids that are central in the relation between wort FAN, ester and higher alcohol concentrations. Procopio *et al.* (2013) studied the significance of individual amino acids on the

higher alcohol and ester concentrations in beer fermented with both lager and ale yeast, and found that the most important amino acids affecting the amount of aroma-active compounds produced during fermentation were leucine, isoleucine, valine, glutamine, cysteine, and proline for *S. pastorianus* and leucine, isoleucine, valine, histidine, glutamine, and proline for *S. cerevisiae*. The results confirm that concentrations of branched-chain amino acids are central in the relation between wort FAN, ester and higher alcohol concentrations, but also suggest that other amino acids influence the production of these aroma compounds as well.

2.4.3 Measuring quality

There exist several collections of, mostly overlapping, standard methods for measuring beer quality, such as the ones published by the European Brewery Convention, American Society of Brewing Chemists, Institute of Brewing & Distilling, and the Brewery Convention of Japan. Quality assessment, in the form of e.g. chemical, microbiological or sensory analysis, is performed at various stages in the brewing process, beginning at the raw materials and ending with the packaged beer, to ensure consistent and high quality. Yeast health and microbiological cleanliness is of particular concern. Typical chemical analysis performed on wort and beer is of original gravity, specific gravity, pH, ethanol content, bitterness, color, FAN, and vicinal diketones. Chemical and microbiological analysis may yield accurate data on the chemical composition and properties of the beer, but these may not necessarily translate into the flavours experienced and appreciated by the consumer. Hence, breweries typically employ the use of sensory analysis for quality assessment as well (Bamforth, 2008; Briggs, 2004).

Sensory analysis, i.e. the use of human senses to analyse the beer, is typically performed by a panel of trained assessors, but consumers may be included as well. Typical tests include comparison tests, triangle tests, determination of flavour thresholds, flavour descriptions, and ranking. The main goal is usually to describe a product or distinguish between several products. A magnitude of flavours can be found in beer, and flavour lists or charts, such as the one by the European Brewery Convention (2008), containing 122 different flavours typically found in beer, or the

one by Dredge (2013), presented in Figure 3, may be employed to aid the panellists. Sensory analysis is typically performed in neutral and quiet environments, using randomized 50-100 ml samples of beer served at 8 or 12° C. The number of panellists or assessments should be sufficiently large to obtain statistical significance, with description analysis requiring a larger amount of assessors. Compared to chemical analysis, sensory analysis is less reliable though, suggesting that constant training and maintenance of panellists are required for accurate results (Briggs, 2004; European Brewery Convention, 2008).

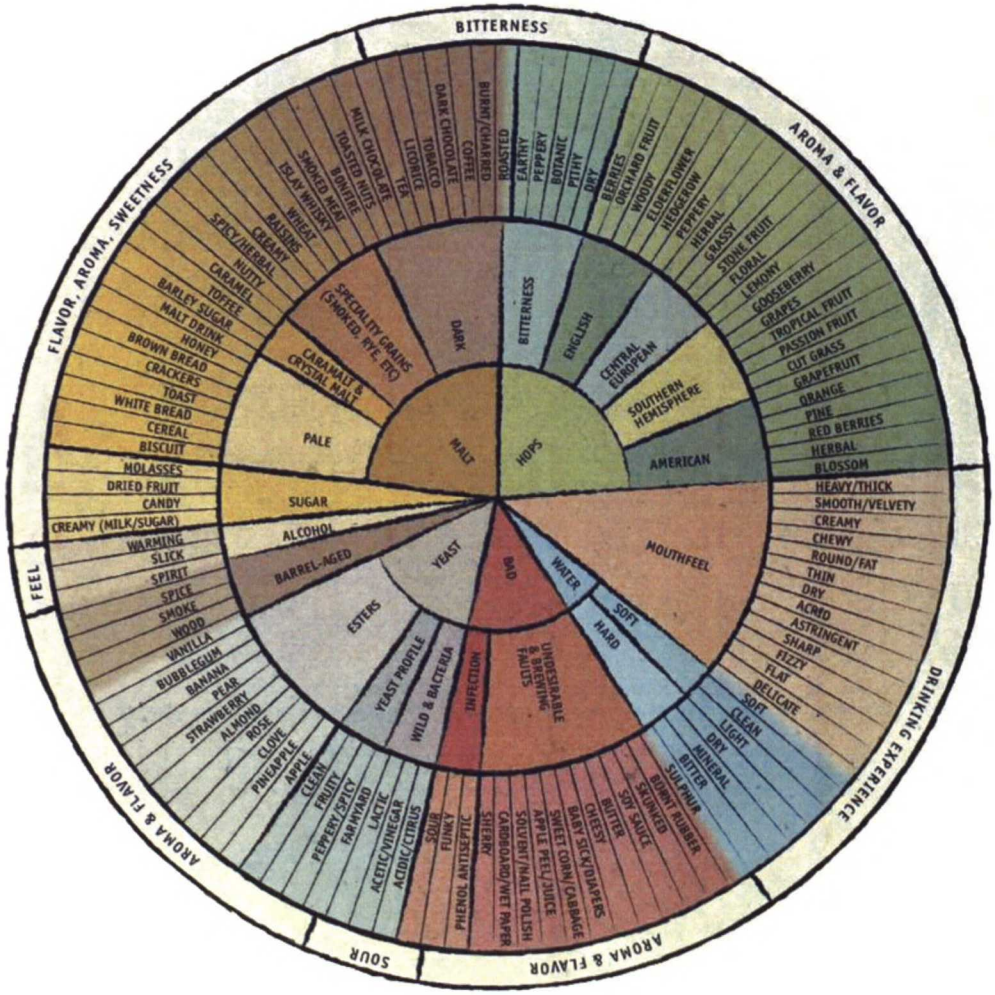

Figure 3 – A chart of flavours and aroma typically found in beer. (Dredge, 2013)

3 Materials and Methods

3.1 Reagents

The reagents, and their manufacturers, used for the experimental work of this thesis are presented in Table 6.

Table 6 – List of reagents used for experimental work.

Name	Description	Manufacturer
MQ-H_2O	Deionized water filtered through ion exchange and activated carbon columns	Millipore S.A., France
Amino acids		
Alanine	L-Alanine, Reagent Grade ≥98%	Sigma-Aldrich, Finland
Arginine	L-Arginine, Reagent Grade ≥98%	Sigma-Aldrich, Finland
Asparagine	L-Asparagine, Reagent Grade ≥98%	Sigma-Aldrich, Finland
Aspartic acid	L-Aspartic acid, Reagent Grade ≥98%	Sigma-Aldrich, Finland
Glutamic acid	L-Glutamic acid, ReagentPlus® ≥99%	Sigma-Aldrich, Finland
Glutamine	L-Glutamine, BioUltra ≥99.5%	Sigma-Aldrich, Finland
Glycine	ReagentPlus® ≥99%	Sigma-Aldrich, Finland
Histidine	L-Histidine, ReagentPlus® ≥99%	Sigma-Aldrich, Finland
Isoleucine	L-Isoleucine, Reagent Grade ≥98%	Sigma-Aldrich, Finland
Leucine	L-Leucine, Reagent Grade ≥98%	Sigma-Aldrich, Finland
Lysine	L-Lysine, Reagent Grade ≥98%	Sigma-Aldrich, Finland
Methionine	DL-Methionine, ≥99%	Sigma-Aldrich, Finland
Phenylalanine	L-Phenylalanine, Reagent Grade ≥98%	Sigma-Aldrich, Finland
Proline	L-Proline, ReagentPlus® ≥99%	Sigma-Aldrich, Finland
Serine	L-Serine, ReagentPlus® ≥99%	Sigma-Aldrich, Finland
Threonine	L-Threonine, Reagent Grade ≥98%	Sigma-Aldrich, Finland
Tryptophan	L-Tryptophan, Reagent Grade ≥98%	Sigma-Aldrich, Finland
Tyrosine	L-Tyrosine, Reagent Grade ≥98%	Sigma-Aldrich, Finland
Valine	L-Valine, Reagent Grade ≥98%	Sigma-Aldrich, Finland
Carbohydrates		
Maltose	D(+)-maltose monohydrate, ≥95%	Sigma-Aldrich, Finland
Glucose	D(+)-glucose	VWR, USA
Fructose	D(-)-fructose, ≥99%	Merck KGaA, Germany
Sucrose	D(+)-saccharose	VWR, USA

Table 6 (cont.) – List of reagents used for experimental work

Mash and Kettle Stabilisers		
$CaSO_4$	Calcium sulphate dihydrate, ≥98%	Sigma-Aldrich, Finland
$CaCl_2$	Calcium chloride dihydrate, ≥99%	Sigma-Aldrich, Finland
$ZnSO_4$	Zinc sulphate heptahydrate, ≥99%	Sigma-Aldrich, Finland
Lactic Acid	(S)-lactic acid, about 90%	Merck KGaA, Germany
Yeast growth medium		
YM	Yeast extract 3 g/l, malt extract 3 g/l, glucose 10 g/l, bacterial agar 15 g/l, peptone 5 g/l	Sigma-Aldrich, Finland
YP	Yeast extract 10 g/l, peptone 20 g/l	Sigma-Aldrich, Finland
Other		
EDTA	Ethylenediaminetetraacetic acid	Sigma-Aldrich, Finland
Reagent Y100	Lysis Buffer	ChemoMetec, Denmark

3.2 Experiment description

A total of four experiments were carried out for this thesis, all focusing on the effect of modifying the wort amino acid profile on the production of vicinal diketones during fermentation. The first three experiments were conducted at a 2-litre scale, while the final experiment was conducted at a 10-litre scale. The first experiment investigated the effect of supplementing various amounts of valine (100, 200 and 300 mg/L) to the wort, the second investigated the effect of supplementing valine (300 mg/L) to worts with standard (407.6 ppm) and reduced FAN content (203.8 ppm), the third investigated the effects of supplementing various groups of amino acids to the wort (grouped according to assimilation rates), and the fourth investigated the effects of pre-conditioning the yeast in a solution containing relatively high concentrations of valine prior to fermentation, on the production of diacetyl and diacetyl precursors and the change of wort valine concentration during fermentation.

3.2.1 Effect of supplementing various amounts of valine to all-malt wort on the production of diacetyl during fermentation

The objective of the first experiment was to compare the effects of various amounts of L-valine supplementation to all-malt wort on diacetyl production during fermentation with a lager yeast strain and investigate whether a relationship exists between wort valine concentration, as well as the ratio of wort valine to other wort amino acids, and the maximum and final wort diacetyl concentration observed during fermentation. L-Valine was supplemented to the worts at three different rates (100, 200 and 300 mg/l), increasing the FAN content of these worts by 12.0, 23.9 and 35.9 ppm α-nitrogen respectively. Density, alcohol by volume, and pH, as well as yeast mass was also monitored during fermentations. Fermentations were carried out in duplicate, and the same yeast strain, fermentation volume, fermentation temperature, fermentation vessel and pitching rate was used for all fermentations. Yeast harvested from a preceding fermentation was used to pitch the experimental fermentations of this experiment. The progress of the experimental fermentations was monitored regularly, through sampling and associated analysis, until fermentation was considered to have ended. A schematic of the fermentations conducted during this experiment is presented in Figure 4.

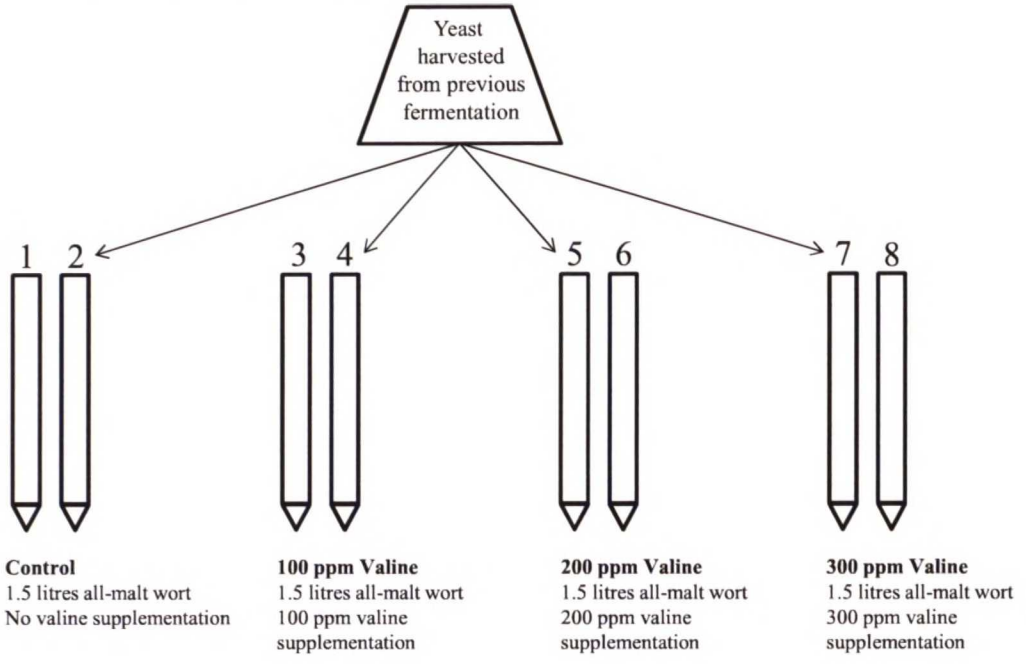

Figure 4 – A schematic of the fermentations conducted during the first experiment.

3.2.1.1 Wort preparation

The all-malt wort (batch number 735) used for all fermentations during the first experiment was prepared at the VTT pilot brewery from barley malt (Scarlett09 pilsner malt, Viking Malt Oy, Finland). The malt was milled with a hammermill (Hamermolen Mono 8, Werkhuizen Schepens Nv, Belgium), sieved through a 2.25 mm sieve, and then mixed with water and stabilisers ($CaCl_2$, $CaSO_4$, and lactic acid) in the mash tun. Mashing was performed using a pre-programmed mashing temperature programme, with 30 minute rests at 48° C, 63° C and 72° C, and a 10 minute rest at 78° C. After complete saccharification of the starch, the mash was pumped through a Meura 2001 mash filter (Meura S.A., Belgium) into the boil kettle. The filtered grains were then sparged with water that was added to the boil kettle. The wort was boiled together with hop CO_2-extract and $ZnSO_4$ for 60 minutes, after which the wort was pumped to a whirlpool kettle for 30 minutes. The warm wort was then transferred into sterile kegs, and kept at 0° C until use. The wort was analysed for specific gravity, pH, FAN, and EBU at VTT according to standard methods described in EBC-Analytica (European Brewery Convention, 2008). The results of this analysis are presented in Table 2 in Appendix 1.

L-Valine was supplemented to the worts used in the experimental fermentations by adding a stock solution. 200 ml of a L-valine solution (target concentration 30 g/l) was prepared by adding 6 g of L-valine to a 200 ml volumetric flask, and then adding MQ-H_2O. The solution was sterile-filtered (0.22µm CA Filter System, Corning Inc., USA), and stored at 4° C until use.

3.2.1.2 Yeast propagation

The yeast used for all the fermentations of this thesis was *Saccharomyces pastorianus* strain VTT-A63015 (VTT Culture Collection, Finland), from here on known as A15. The yeast was obtained from a less than one month old pure culture grown on an YM agar plate for approximately 24 hours at room temperature and then stored at under 4° C. Two loopfuls of the yeast was inoculated from the YM agar plate into 150 ml of YP medium (4% maltose) prepared in a 250 ml Erlenmeyer flask

using a sterile inoculating loop. The YP medium (4% maltose) was prepared by mixing 75 ml of double-strength YP medium and 75 ml of an 8% (w/v) maltose solution. After inoculation, the flask was placed on an orbital shaker (TR-125, Infors AG, Switzerland) set to 160 RPM, and allowed to grow for 44 hours at 25° C.

After the flask was removed from the orbital shaker, the optical density of the yeast suspension was measured. 20 µl of yeast suspension was diluted with 2 ml of a 10 mM EDTA solution in a cuvette, after which the mixture was thoroughly mixed with a vortex mixer (Vortex-Genie 2, Scientific Instruments Inc., USA) and its absorbance at 600 nm was measured with a spectrophotometer (Shimadzu UV-1800, Shimadzu Corp., Japan) using the 10 mM EDTA solution as a blank. The yeast suspension was then used to inoculate 3 l of wort 732, which had been aseptically transferred from a keg into a 5 l Erlenmeyer flask, to an optical density of 0.15. The amount of yeast suspension (V_{sus}) needed for inoculation was calculated from its optical density (OD_{sus}) according to Equation (1):

$$V_{sus} = \frac{3000\ \text{mL} \cdot 0.15}{OD_{sus} - 0.15} \tag{1}$$

The inoculated wort was then aseptically split into two 3 l Erlenmeyer flasks, and the flasks were placed on an orbital shaker set to 100 RPM, and allowed to grow for 90 hours at 18° C.

The flasks were then transferred to a cold room (4° C) for 24 hours, to allow the yeast to sediment. A 40 ml sample was drawn from both flasks, after which the samples were centrifuged at 9000 RPM for 10 minutes at 2° C (Sigma 4K 15 with Sigma 12166 rotor, Sigma Laborzentrifugen Gmbh, Germany), and the specific gravity of the supernatants was measured with a density meter (see section 3.3) to ensure fermentation had occurred. A yeast slurry was then prepared from the sedimented yeast, by first pouring off the majority of the supernatant wort of both flasks into a 5 l Erlenmeyer flask, and then combining the two yeast slurries in a pre-weighed flask. Two 2 ml samples were taken from the yeast slurry, and placed in pre-weighed centrifuge tubes. The centrifuge tubes, containing the samples, were

weighed (Mettler-Toledo AT 200, Mettler-Toledo Inc., Switzerland), then the samples were centrifuged at 9000 RPM for 10 minutes, after which the supernatant was poured off and the yeast pellets were washed with 25 ml MQ-H_2O and centrifuged again at 9000 RPM for 10 minutes. The supernatant was again poured off, and the centrifuge tubes, containing yeast pellets, were weighed. The mass differences were then used to calculate the concentration of yeast in the slurry. The yeast slurry was diluted to 20% (w/w) using the collected supernatant wort. A first fermentation (Generation 0) was then initiated by aseptically pitching 200 g of the yeast slurry (for a pitching rate of 5 g/l) into 8.489 kg (8 litres) of wort 732 (oxygenated to 8.5 ppm oxygen content (Oxygen Indicator model 26073 and sensor 21158, Orbisphere laboratories, Switzerland)) in a 10-litre tall-tube cylindroconical fermentation vessel. The fermentation was carried out at 15° C for 6 days.

The yeast (*S. pastorianus* A15) used for the first experiment was harvested from the beers that were collected from a fourth generation fermentation of the previously propagated yeast. A yeast slurry was prepared from the sedimented yeast in the beer, by first decanting the beer into 1 l Erlenmeyer flasks, and then combining the two yeast slurries in a pre-weighed flask. The concentration of yeast in the slurry was determined as described above. The yeast slurry was diluted to 25% (w/w) using the collected decanted beer. The viability of the yeast was determined using an integrated fluorescence microscope according to the method depicted in Section 3.3.3. The yeast slurry was then used to inoculate the experimental fermentations.

3.2.1.3 Fermentation

The experimental fermentation was initiated by aseptically pitching 30 g of the 25% yeast slurry (for a pitching rate of 5 g/l) into eight 2-litre cylindroconical fermentation vessels. Before use, the vessels were washed and cleaned using a 2% chlorine-based alkaline cleaning agent solution (P3-ansep® ALU, Ecolab Inc., USA), after which they were sanitized using a 0.5% hydrogen peroxide/peracetic acid-based acidic disinfectant solution (P3-oxonia active®, Ecolab Inc., USA). The yeast slurry was mixed with the valine solution 30 minutes prior to pitching, after which it was added to the fermentation vessels first, followed by wort 735 (oxygenated to 9 ppm

oxygen content (Oxygen Indicator model 26073 and sensor 21158, Orbisphere laboratories, Switzerland)) according to Table 7. The target fermentation volume for all fermentations was 1.5 l. The fermentations were carried out at 15° C for 8 days. Samples were regularly drawn from the fermentation vessels, and they were tested for density, ethanol content, pH, yeast fresh mass, yeast dry mass, and vicinal diketones (see section Section 3.3). If samples weren't analysed directly, they were frozen and kept at -20° C until analysis. After fermentation, the beer from each fermentation vessel was collected in sterile 1 l Erlenmeyer flasks, which were then transferred to a cold room (4° C) for storage.

Table 7 – Composition of worts used in experimental fermentations.

Fermentation # and name	**All-malt wort 735**		**L-Valine**	
	litres	**kg**	**mg**	**ml solution**
Control A	1.5	1.589	-	-
Control B	1.5	1.589	-	-
100ppm Valine A	1.5	1.589	150	5
100ppm Valine B	1.5	1.589	150	5
200ppm Valine A	1.5	1.589	300	10
200ppm Valine B	1.5	1.589	300	10
300ppm Valine A	1.5	1.589	450	15
300ppm Valine B	1.5	1.589	450	15

3.2.2 Effects of valine supplementation and FAN content of wort on the production of diacetyl during fermentation

The objective of the second experiment was to compare the effects of L-valine supplementation to all-malt wort (standard FAN content) and semi-synthetic wort (low FAN content) on diacetyl production during fermentation with a lager yeast strain. L-Valine was supplemented to the worts at a rate of 300 mg/l, increasing the FAN content of these worts by 35.9 ppm α-nitrogen. Density, alcohol by volume, pH, and amino acid profile of the wort, as well as yeast mass was also monitored during fermentations. Fermentations were carried out in duplicate, and the same yeast strain, fermentation volume, fermentation temperature, fermentation vessel and pitching rate was used for all fermentations. It was also attempted to match the concentration of fermentable sugars in the all-malt wort and the semi-synthetic wort. Yeast harvested from the Generation 0 fermentation, performed on all-malt wort using similar environmental conditions to those of the experimental fermentation, was then used to pitch the actual experimental fermentations. This was done to mimic the physiological state of yeast cells in actual brewery fermentations, where re-pitching of yeast occurs for several generations. The progress of the experimental fermentations was monitored regularly, through sampling and associated analysis, until fermentation was considered to have ended. A schematic of the fermentations conducted during this experiment is presented in Figure 5.

3.2.2.1 Wort preparation

The all-malt wort (batch number 732) used for all fermentations during the second experiment was prepared at the VTT pilot brewery according to method used to produce batch number 735, described in section 3.2.1.1. The wort was analysed for specific gravity, pH, FAN, EBU, and Apparent Attenuation Limit at VTT according to standard methods described in EBC-Analytica (European Brewery Convention, 2008). The results of this analysis are presented in Table 1 in Appendix 1.

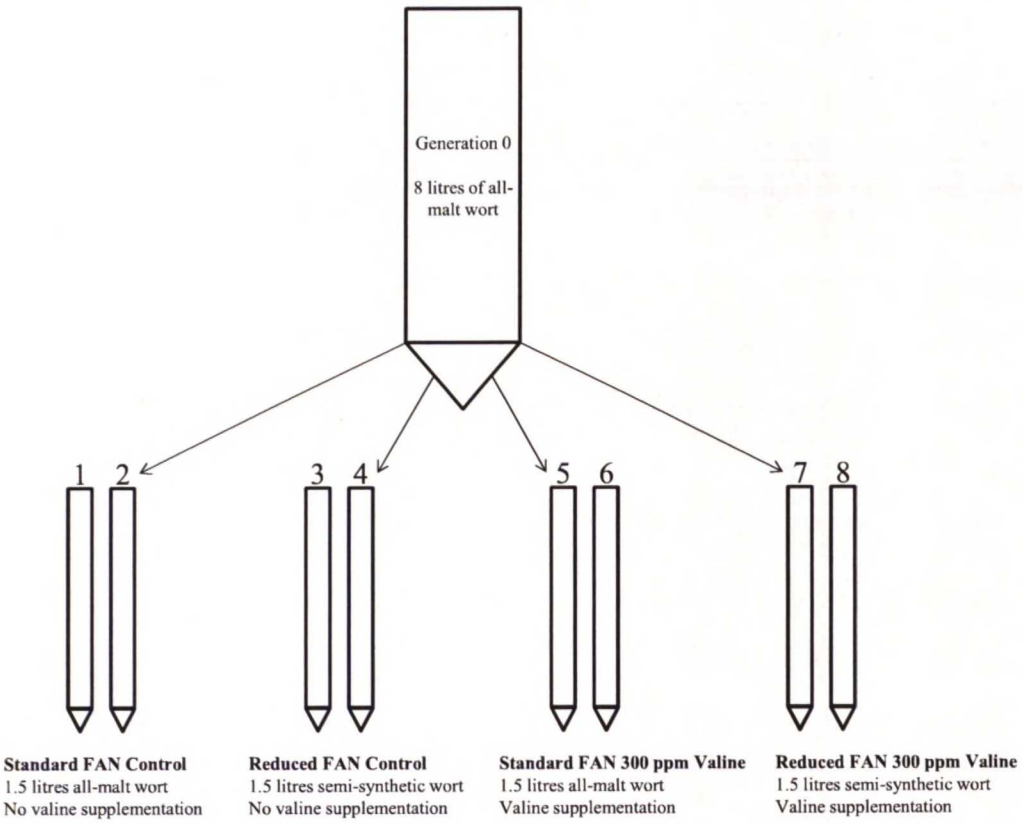

Figure 5 – A schematic of the fermentations conducted during the first experiment.

The semi-synthetic wort used in the experimental fermentations was prepared by diluting all-malt wort 732 with an equal volume of sugar solution, containing the same concentration of fermentable sugars as the all-malt wort. The concentrations of fermentable sugars in all-malt wort 732 were determined by HPAEC (see section 3.3.9) and are presented in Table 8, while the concentrations of amino acids in the wort was determined using HPLC (see section 3.3.7) and are presented in Table 9. For the sugar solution, maltotriose was replaced by an equal amount of maltose due to cost. 3500 ml of the sugar solution was prepared by first adding 66.5 g glucose, 16.45 g fructose, and 304.5 g maltose to a 3000 ml volumetric flask, which was then filled with MQ-H_2O. The solution was distributed into smaller containers and autoclaved at 121° C for 20 minutes. 5.25 g sucrose was added to a 500 ml volumetric flask, which was then filled with MQ-H_2O. This solution was sterile-filtered (0.22µm CA Filter System, Corning Inc., USA). The solutions were allowed to cool overnight at room temperature and then combined in a laminar flow cabinet

the following morning. The density of this solution was measured with a density meter (Anton Paar Density Meter DMA 5000 M, Anton Paar GmbH, Austria), to allow for adding sugar solution to fermenting vessels by mass.

Table 8 – The concentrations of fermentable sugars in all-malt wort 732.

Sugar	Concentration (g/l)
Glucose	19
Fructose	4.7
Sucrose	1.5
Maltose	68
Maltotriose	19

L-Valine was supplemented to the worts used in the experimental fermentations by adding the stock solution (containing 30 g L-valine / l) produced in the first experiment (see section 3.2.1.1). The solution was stored at 4° C since previous use.

3.2.2.2 Yeast propagation

Beer from the Generation 0 fermentation (described in Section 3.2.1.2) was collected in two 5 l Erlenmeyer flasks, which were then placed in a cold room (4° C) for 24 hours, to allow the yeast to sediment. A yeast slurry was then prepared from the sedimented yeast, by first decanting off beer from both flasks into a 5 l Erlenmeyer flask, and then combining the two yeast slurries in a pre-weighed flask. Two 2 ml samples were taken from the yeast slurry, and placed in pre-weighed centrifuge tubes, and the concentration of yeast in the slurry was determined as described in Section 3.2.1.2. The yeast slurry was diluted to 30% (w/w) using the collected supernatant wort. The viability of the yeast was determined using an integrated fluorescence microscope according to the method depicted in Section 3.3.3. The yeast slurry was then used to inoculate the experimental fermentations.

Table 9 – The concentrations of amino acids in all-malt wort 732.

Amino acid	Concentration (µmol/l)	Concentration (mg/l)
Alanine	2788	248
Arginine	1742	303
Asparagine	1593	210
Aspartic acid	968	129
Glutamine	375	54.8
Glutamic acid	703	103
Glycine	1098	82.5
Histidine	637	98.8
Isoleucine	1229	161
Leucine	2900	380
Lysine	1734	253
Methionine	457	68.1
Phenylalanine	1714	283
Proline	6536	752
Serine	1394	146
Threonine	1119	133
Tryptophan	378	77.3
Tyrosine	1509	273
Valine	2251	264

3.2.2.3 Fermentation

The experimental fermentation was initiated by aseptically pitching 25 g of the 30% yeast slurry (for a pitching rate of 5 g/l) into eight 2-litre cylindroconical fermentation vessels. Before use, the vessels were washed and cleaned using a 2% chlorine-based alkaline cleaning agent solution (P3-ansep® ALU, Ecolab Inc., USA), after which they were sanitized using a 0.5% hydrogen peroxide/peracetic acid-based acidic disinfectant solution (P3-oxonia active®, Ecolab Inc., USA). The yeast slurry was added to the fermentation vessels first, followed by the L-valine solution, wort

732 (oxygenated to 9 ppm oxygen content (Oxygen Indicator model 26073 and sensor 21158, Orbisphere laboratories, Switzerland)), and the sugar solution (replicating the wort sugar profile; also oxygenated to 9 ppm oxygen content) according to Table 10. The target fermentation volume for all fermentations was 1.5 l. The fermentations were carried out at 15° C for 8 days. Samples were regularly drawn from the fermentation vessels, and they were tested for density, ethanol content, pH, yeast fresh mass, yeast dry mass, vicinal diketones and amino acids (see section 3.3). If samples weren't analysed directly, they were frozen and kept at -20° C until analysis. After fermentation, the beer from each fermentation vessel was collected in sterile 1 l Erlenmeyer flasks, which were then transferred to a cold room (4° C) for storage.

Table 10 – Composition of worts used in experimental fermentations.

Fermentation # and name	**All-malt wort 732**		**Sugar solution**		**L-Valine**	
	litres	**kg**	**litres**	**kg**	**mg**	**ml solution**
Standard FAN Control A	1.5	1.592	-	-	-	-
Standard FAN Control B	1.5	1.592	-	-	-	-
Reduced FAN Control A	0.75	0.796	0.75	0.783	-	-
Reduced FAN Control B	0.75	0.796	0.75	0.783	-	-
Standard FAN 300 ppm Valine A	1.5	1.592	-	-	450	15
Standard FAN 300 ppm Valine B	1.5	1.592	-	-	450	15
Reduced FAN 300 ppm Valine A	0.75	0.796	0.75	0.783	450	15
Reduced FAN 300 ppm Valine B	0.75	0.796	0.75	0.783	450	15

3.2.3 Effect of altering the amino acid spectrum of wort on the production of diacetyl during fermentation

The objective of the third experiment was to compare the effects of supplementing amino acids from three different groups (named preferred amino acids (PAA), non-preferred amino acids (NPAA), and branched-chain amino acids (BCAA)) to an all-malt wort (standard FAN content) and a semi-synthetic wort (low FAN content) on diacetyl production during fermentation with a lager yeast strain. Density, alcohol by volume, pH, and amino acid profile of the wort, as well as yeast mass was also monitored during fermentations. The amino acids were grouped into the three groups based on their absorption rates and structures. Absorption rates were determined utilizing unpublished data from previous studies on the change of amino acid concentrations during fermentation of wort 732 with the A15 yeast strain (see Table 1 in Appendix 2). The first group, preferred amino acids (PAA), contains the amino acids which had a higher uptake rate than valine during the first 25 hours of fermentation (see Table 11). The second group, non-preferred amino acids (NPAA), contains the amino acids which had a lower uptake rate than valine during the first 25 hours of fermentation (see Table 12). The third group, branched-chain amino acids (BCAA), contains leucine and isoleucine, which have a similar structure to valine.

Table 11 – The amino acids in the preferred amino acids (PAA) group.

Abbreviation	Amino acid
Asn	Asparagine
Ser	Serine
Thr	Threonine
Leu	Leucine
Lys	Lysine
Pro	Proline
Arg	Arginine
Phe	Phenylalanine
Gln	Glutamine
Asp	Aspartic acid

Table 12 – The amino acids in the non-preferred amino acids (NPAA) group.

Abbreviation	Amino acid
Ile	Isoleucine
His	Histidine
Gly	Glycine
Glu	Glutamic acid
Trp	Tryptophan
Ala	Alanine
Met	Methionine
Tyr	Tyrosine

Fermentations were carried out in duplicate, using the same yeast strain, fermentation volume, fermentation temperature, fermentation vessel and pitching rate. An attempt was made to match the concentration of fermentable sugars in the all-malt wort and the semi-synthetic wort, as well as the valine content. Hence, valine was supplemented to the semi-synthetic worts to raise the concentration of valine to that in the all-malt wort. Yeast harvested from the fermentations of the second experiment was used to propagate yeast for the experimental fermentations of this experiment. After the experimental fermentations were carried out, it was observed that the addition of the PAA solution raised the wort pH significantly, so control and PAA fermentations were repeated in a second experimental fermentation. In the repeated fermentations the pH of the worts supplemented with the PAA solution was adjusted using lactic acid. The progress of the experimental fermentations was monitored regularly, through sampling and associated analysis, until fermentation was considered to have ended. A schematic of the fermentations conducted during this experiment is presented in Figure 6.

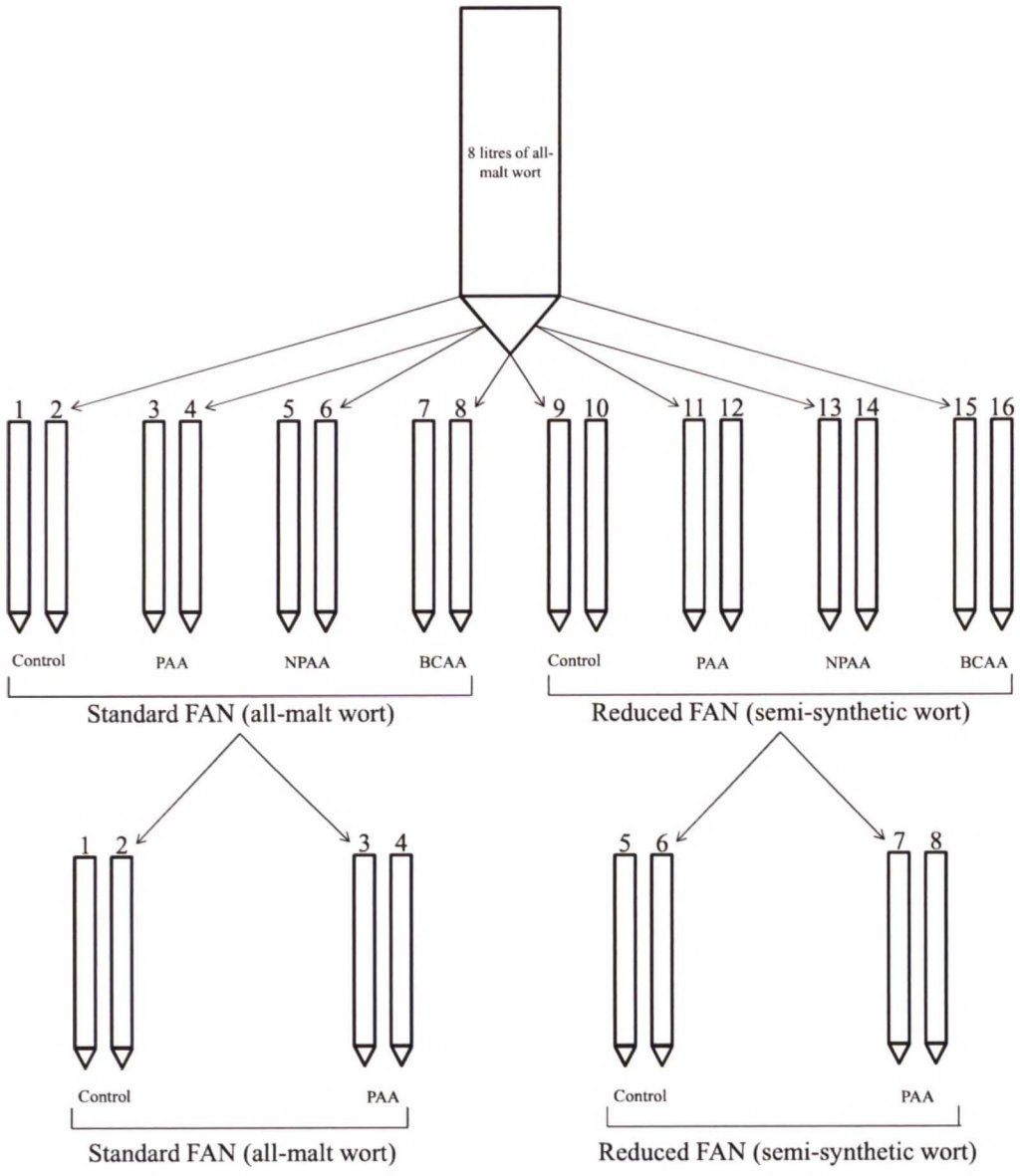

Figure 6 – A schematic of the fermentations conducted during the second experiment.

3.2.3.1 Wort preparation

The all-malt wort (batch number 732) used for all fermentations during the second experiment was prepared at the VTT pilot brewery as described in section 3.2.1.1. The semi-synthetic wort used in the experimental fermentations was prepared as in the first experiment by diluting all-malt wort 732 with an equal volume of sugar solution, containing the same concentration of fermentable sugars as the all-malt wort (see Table 7 in section 3.2.1.1). For the sugar solution, maltotriose was again replaced with maltose, but for this experiment only a third of the maltotriose was replaced with maltose due to the lower fermentability of maltotriose. 7000 ml of the sugar solution was prepared by first adding 133 g glucose, 32.9 g fructose, and 520.3 g maltose to a 5000 ml volumetric flask, which was then filled with MQ-H_2O. The solution was distributed into smaller containers and autoclaved at 121° C for 20 minutes. 10.5 g sucrose was added to a 2000 ml volumetric flask, which was then filled with MQ-H_2O. This solution was sterile-filtered (0.22µm CA Filter System, Corning Inc., USA). The solutions were allowed to cool overnight at room temperature and then combined in a laminar flow cabinet the following morning. The density of this solution was measured with a density meter (Anton Paar Density Meter DMA 5000 M, Anton Paar GmbH, Austria), to allow for adding sugar solution to fermenting vessels by mass.

The amino acids were supplemented to the worts from stock solutions containing amino acids at concentrations 15 times larger than in all-malt wort 732 (see Table 9 in section 3.2.2.1). The concentration of tyrosine in the NPAA stock solution was only 1.5 times larger than in all-malt wort 732, because of the low solubility of tyrosine in water at 25° C (0.4474 g/l; Hitchcock, 1924). 500 ml of each amino acid group solution was made by adding the amounts presented in Tables 13-15 (PAA, NPAA and BCAA solutions respectively) to a 500 ml volumetric flask. The flasks were then filled with MQ-H_2O and the solutions were sterile-filtered (0.22µm CA Filter System, Corning Inc., USA), and stored at 4° C until use.

Table 13 – The amount of amino acids added to the 500 ml volumetric flask for the creation of the preferred amino acid (PAA) stock solution.

Amino acid	Target concentration (g/l)	Mass added to 500 ml flask (g)
Asparagine	3.2	1.58
Serine	2.2	1.10
Threonine	2.0	1.00
Leucine	5.7	2.85
Lysine	3.8	1.90
Proline	11.3	5.64
Arginine	4.6	2.28
Phenylalanine	4.2	2.12
Glutamine	0.8	0.41
Aspartic acid	1.9	0.97

Table 14 – The amount of amino acids added to the 500 ml volumetric flask for the creation of the non-preferred amino acid (NPAA) stock solution.

Amino acid	Target concentration (g/l)	Mass added to 500 ml flask (g)
Isoleucine	2.4	1.21
Histidine	1.5	0.74
Glycine	1.2	0.62
Glutamic acid	1.6	0.78
Tryptophan	1.2	0.58
Alanine	3.7	1.86
Methionine	1.0	0.51
Tyrosine	0.41	0.21

Table 15 – The amount of amino acids added to the 500 ml volumetric flask for the creation of the branched-chain amino acid (BCAA) stock solution.

Amino acid	Target concentration (g/l)	Mass added to 500 ml flask (g)
Isoleucine	2.4	1.21
Leucine	5.7	2.85

L-Valine was supplemented to the semi-synthetic worts used in the experimental fermentations by adding a stock solution. 100 ml of a L-valine solution (target concentration 19.8 g/l, i.e. 75 times larger than in all-malt wort 732) was prepared by adding 1,98 g of L-valine to a 100 ml volumetric flask, and then adding MQ-H_2O. The solution was sterile-filtered (0.22µm CA Filter System, Corning Inc., USA), and stored at 4° C until use.

3.2.3.2 Yeast propagation

Lager yeast (*S. pastorianus* A15) was harvested from the Standard FAN Control beers that were collected from the fermentations of the second experiment. A yeast slurry was prepared from the sedimented yeast in the beer, by first decanting off the beer of both flasks into a 1 l Erlenmeyer flask, and then combining the two yeast slurries in a pre-weighed flask. The concentration of yeast in the slurry was determined as in Section 3.2.1.2. The yeast slurry was diluted to 20% (w/w) using the decanted beer. The viability of the yeast was determined using an integrated fluorescence microscope according to the method depicted in Section 3.3.3. A first fermentation was then initiated by aseptically pitching 200 g of the yeast slurry (for a pitching rate of 5 g/l) into 8.489 kg (8 litres) of wort 732 (oxygenated to 10 ppm oxygen content (Oxygen Indicator model 26073 and sensor 21158, Orbisphere laboratories, Switzerland)) in a 10-litre tall-tube cylindroconical fermentation vessel. The fermentation was carried out at 15° C for 8 days.

After the first fermentation had finished, the beer was collected in two 5 l Erlenmeyer flasks, which were then placed in a cold room (4° C) for 24 hours, to allow the yeast to sediment. A yeast slurry was then prepared from the sedimented

yeast, by decanting off the beer of both flasks into a 5 l Erlenmeyer flask, and then combining the two yeast slurries in a pre-weighed flask. The concentration of yeast in the slurry was determined as in Section 3.2.1.2. The yeast slurry was diluted to 25% (w/w) using the collected supernatant wort. The viability of the yeast was determined using an integrated fluorescence microscope according to the method depicted in Section 3.3.3. The yeast slurry was then used to inoculate the experimental fermentations.

3.2.3.3 Fermentation

The experimental fermentation was then initiated by aseptically pitching 32 g of the 25% yeast slurry (for a pitching rate of 5 g/l) into sixteen 2-litre cylindroconical fermentation vessels. Before use, the vessels were washed and cleaned using a 2% chlorine-based alkaline cleaning agent solution (P3-ansep® ALU, Ecolab Inc., USA), after which they were sanitized using a 0.5% hydrogen peroxide/peracetic acid-based acidic disinfectant solution (P3-oxonia active®, Ecolab Inc., USA). The yeast slurry was added to the fermentation vessels first, followed by half the wort amount (all-malt wort 732 and the sugar solution, both oxygenated to 9 ppm oxygen content (Oxygen Indicator model 26073 and sensor 21158, Orbisphere laboratories, Switzerland)), then the amino acid solutions, and finally the rest of the wort, according to Table 16. The target fermentation volume for all fermentations was 1.6 l. The fermentations were carried out at 15° C for 8 days. Samples were regularly drawn from the fermentation vessels, and they were tested for density, ethanol content, pH, yeast fresh mass, yeast dry mass, vicinal diketones, amino acids and aroma compounds (see section Section 3.3). If samples weren't analysed directly, they were frozen and kept at -20° C until analysis. After fermentation, the beer from each fermentation vessel was collected in sterile 1 l Erlenmeyer flasks, which were then transferred to a cold room (4° C) for storage.

For the second experimental fermentation, a 25% yeast slurry was prepared from beer collected from the first experimental fermentations as described in Section 3.2.3.2. The second experimental fermentation was initiated aseptically pitching 32 g of the 25% yeast slurry (for a pitching rate of 5 g/l) into eight 2-litre cylindroconical

fermentation vessels. Pitching, fermentations, and sampling were carried out as above, except that lactic acid was added to the PAA-supplemented worts prior to adding them to the fermenting vessel. The composition of the worts used for the second experimental fermentations is presented in Table 17.

Table 16 – Composition of worts used for the first experimental fermentations.

#	Wort 732		Sugar Solution		PAA solution	NPAA solution	BCAA solution	Valine solution	Sterile water
	l	kg	l	kg	ml	ml	ml	ml	ml
1	1.5	1.592	-	-	-	-	-	-	100
2	1.5	1.592	-	-	-	-	-	-	100
3	1.5	1.592	-	-	100	-	-	-	-
4	1.5	1.592	-	-	100	-	-	-	-
5	1.5	1.592	-	-	-	100	-	-	-
6	1.5	1.592	-	-	-	100	-	-	-
7	1.5	1.592	-	-	-	-	100	-	-
8	1.5	1.592	-	-	-	-	100	-	-
9	0.75	0.796	0.75	0.779	-	-	-	10	90
10	0.75	0.796	0.75	0.779	-	-	-	10	90
11	0.75	0.796	0.75	0.779	50	-	-	10	40
12	0.75	0.796	0.75	0.779	50	-	-	10	40
13	0.75	0.796	0.75	0.779	-	50	-	10	40
14	0.75	0.796	0.75	0.779	-	50	-	10	40
15	0.75	0.796	0.75	0.779	-	-	50	10	40
16	0.75	0.796	0.75	0.779	-	-	50	10	40

Table 17 – Composition of worts used for the second experimental fermentations.

#	Wort 732		Sugar Solution		PAA solution	Valine solution	Sterile water	90% Lactic acid
	l	kg	l	kg	ml	ml	ml	µl
1	1.5	1.592	-	-	-	-	100	-
2	1.5	1.592	-	-	-	-	100	-
3	1.5	1.592	-	-	100	-	-	320
4	1.5	1.592	-	-	100	-	-	320
5	0.75	0.796	0.75	0.779	-	10	90	-
6	0.75	0.796	0.75	0.779	-	10	90	-
7	0.75	0.796	0.75	0.779	50	10	40	260
8	0.75	0.796	0.75	0.779	50	10	40	260

3.2.4 Effects of supplementing valine to all-malt wort and pre-conditioning yeast in a valine/glucose solution on beer quality and the production of diacetyl during fermentation

As a continuation of the first and second experiment, the objective of the fourth and final experiment was to compare the effects of supplementing L-valine to all-malt wort and pre-conditioning a lager yeast strain in an aerated valine/glucose solution (3 and 6 g/L respectively) on diacetyl production during fermentation and in the matured beer. L-Valine was supplemented to the worts at a rate of 100 mg/l, increasing the FAN content of the wort by 12.0 ppm α-nitrogen. Density, alcohol by volume, and pH, as well as yeast mass was also monitored during fermentations. Fermentations were carried out in duplicate, and the same yeast strain, fermentation volume, fermentation temperature, fermentation vessel and pitching rate was used for all fermentations. Prior to the experimental fermentations, a first fermentation was performed with all-malt wort using similar environmental conditions to those of the experimental fermentations to propagate enough yeast for the experimental fermentations. The progress of the experimental fermentations was monitored regularly, through sampling and associated analysis, until fermentation was considered to have ended. A schematic of the fermentations conducted during this experiment is presented in Figure 7.

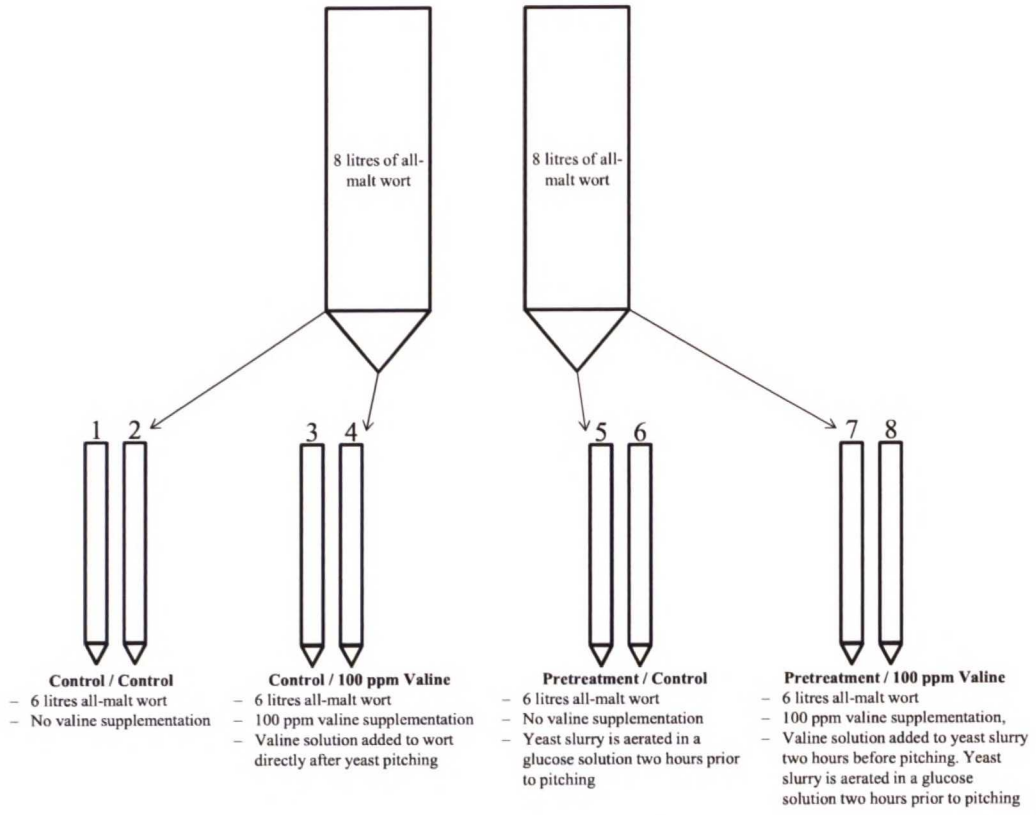

Figure 7 – A schematic of the fermentations conducted during the fourth experiment.

3.2.4.1 Wort preparation

The all-malt wort (batch number 737) used for the experimental fermentations during the fourth experiment was prepared at the VTT pilot brewery according to method used to produce batch number 735, described in section 3.2.1.1. The wort was analysed for specific gravity, pH, FAN, and EBU at VTT according to standard methods described in EBC-Analytica (European Brewery Convention, 2008). The results of this analysis are presented in Table 3 in Appendix 1. L-Valine was supplemented to the worts used in the experimental fermentations by adding the stock solution (containing 30 g L-valine / l) produced in the first experiment (see section 3.2.1.1). The solution was stored at 4° C since previous use.

3.2.4.2 Yeast propagation

Lager yeast (*S. pastorianus* A15) was propagated from a pure culture grown on an YM agar plate in YP medium (4% maltose) and all-malt wort according to the method described in section 3.2.1.2. A yeast slurry was prepared from the sedimented yeast in beer collected from the earlier propagation, by first decanting off the beer of both flasks into a 1 l Erlenmeyer flask, and then combining the two yeast slurries in a pre-weighed flask. The concentration of yeast in the slurry was determined as in Section 3.2.1.2. The yeast slurry was diluted to 30% (w/w) using the collected supernatant beer. The viability of the yeast was determined using an integrated fluorescence microscope according to the method depicted in Section 3.3.3. The first fermentation was then initiated by aseptically pitching 133.3 g of the yeast slurry (for a pitching rate of 5 g/l) into 8.499 kg (8 litres) of wort 732 (oxygenated to 9 ppm oxygen content (Oxygen Indicator model 26073 and sensor 21158, Orbisphere laboratories, Switzerland)) in a 10-litre tall-tube cylindroconical fermentation vessel. The fermentation was carried out at 15° C for 8 days.

After the first fermentation had finished, the beer was collected in four 5 l Erlenmeyer flasks, which were then placed in a cold room (0° C) for 24 hours, to allow the yeast to sediment. A yeast slurry was then prepared from the sedimented yeast, by first pouring off the majority of the supernatant wort of both flasks into a 5 l Erlenmeyer flask, and then combining the two yeast slurries in a pre-weighed flask. The concentration of yeast in the slurry was determined as in Section 3.2.1.2. The yeast slurry was diluted to 30% (w/w) using the collected supernatant wort. The viability of the yeast was determined using an integrated fluorescence microscope according to the method depicted in Section 3.3.3. The yeast was pre-conditioned at a concentration of 15% in an aerated and constantly agitated solution containing 6 g glucose. The valine-supplemented pre-treatment solution contained 3 g valine per litre as well. Pre-conditioning was carried out at a temperature of 10° C for 2 hours. The control yeast, i.e. those that were not pre-conditioned, was kept at 0° C until pitching. A summary of the composition of the pre-treatment solution and treatment conditions are presented in Table 18.

Table 18 – Summary of the compositions of the pre-treatment solutions and the treatment conditions.

Parameter	Control (no pre-treatment)		Pre-treatment	
	Control	100 ppm Valine	Control	100 ppm Valine
30% yeast slurry (ml)	250	250	250	250
Sterile MQ-H_2O (ml)	250	250	0	0
30 g/L valine (ml)	0	0	0	50
12 g/L glucose (ml)	0	0	250	0
15 g/L glucose (ml)	0	0	0	200
Aeration (ml/min)	0	0	30	30
Temperature (° C)	0	0	10	10
Agitation	No	No	Yes	Yes
Time (h)	0	0	2	2

3.2.4.3 Fermentation

The experimental fermentation was initiated by aseptically pitching 200 g of the 15% yeast slurry (for a pitching rate of 5 g/l) into eight 10-litre tall-tube cylindroconical fermentation vessels. Before use, the vessels were washed and cleaned using a 2% chlorine-based alkaline cleaning agent solution (P3-ansep® ALU, Ecolab Inc., USA), after which they were sanitized using a 0.5% hydrogen peroxide/peracetic acid-based acidic disinfectant solution (P3-oxonia active®, Ecolab Inc., USA). The yeast slurry was added to the fermentation vessels first, followed by the potential valine solution (20 ml to the Control / 100 ppm Valine fermentations), and 6.356 kg of wort 737 (oxygenated to 12 ppm oxygen content (Oxygen Indicator model 26073 and sensor 21158, Orbisphere laboratories, Switzerland)). The target fermentation volume for all fermentations was 6 l. The fermentations were carried out at 15° C for 8 days. Samples were regularly drawn from the fermentation vessels, and they were tested for density, ethanol content, pH, yeast fresh mass, yeast dry mass, and vicinal diketones (see section Section 3.3). If samples weren't analysed directly, they were frozen and kept at -20° C until analysis. After fermentation, the beer from each fermentation vessel was transferred to sterile kegs (duplicates combined), and allowed to mature at 12° C for 5 days. After this the kegs were transferred to 0° C and the beer was allowed to stabilize for 2 days, after which the beer was filtered

through filter sheets (Seitz K-150, Pall Corporation, USA), diluted to 5% ABV using boiled tap water, and carbonated using 100% carbon dioxide (AGA Suomi, Finland). The beer was then bottled into 33 cl brown glass bottles (DFC1 Filler & Crowner, HDP Brewing Systems, Canada), and bottles were stored at 0° C until foam stability analysis.

3.3 Methods of analysis

3.3.1 Yeast fresh mass

The yeast fresh mass content of samples drawn from the fermentation vessels was determined by first placing samples in pre-weighed 50-ml centrifuge tubes (m_{tube}), and then measuring the mass of the tubes containing the samples ($m_{\text{tube+sample}}$). The tubes, containing the samples, were then centrifuged for 10 minutes at 9000 RPM and 2° C (Sigma 4K 15 with Sigma 12166 rotor, Sigma Laborzentrifugen Gmbh, Germany), after which the supernatant was poured off and collected for other analysis. The remaining yeast pellet was then washed with 25 ml MQ-H_2O, and the tubes were thoroughly mixed using a vortex mixer (Vortex-Genie 2, Scientific Instruments Inc., USA). The tubes were then centrifuged again for 10 minutes at 9000 RPM and 2° C, after which the supernatant was poured off, and the mass of the tubes containing yeast pellets was measured ($m_{\text{tube+yeast}}$). The yeast fresh mass content, $C_{\text{yeast,fresh}}$ (g/l), was then calculated according to Equation (2):

$$C_{\text{yeast,fresh}} = \frac{\left(m_{\text{tube+yeast}} - m_{\text{tube}}\right) \cdot \rho_{\text{wort}}}{\left(m_{\text{tube+sample}} - m_{\text{tube}}\right)} \tag{2}$$

where $m_{\text{tube+yeast}}$ is the mass of the centrifuge tube containing the yeast pellet (g)

m_{tube} is the mass of the empty centrifuge tube (g)

ρ_{wort} is the density of the wort at the time of sampling (g/l)

$m_{\text{tube+sample}}$ is the mass of the centrifuge tube containing the sample (g).

3.3.2 Yeast dry mass

The yeast dry mass content of samples drawn from the fermentation vessels was determined by suspending the yeast pellet gained from yeast fresh mass analysis (section 3.3.1) in a total of 6 ml MQ-H_2O (added to the centrifuge tube in 2 ml additions) and mixing thoroughly with a vortex mixer (Vortex-Genie 2, Scientific Instruments Inc., USA). The suspension was then transferred into a pre-weighed porcelain crucible (m_{crucible}) before the next 2 ml MQ-H_2O addition. The crucible was then dried overnight at 105° C and allowed to cool in a desiccator, before its mass was measured ($m_{\text{crucible+yeast}}$). The yeast dry mass content, $C_{\text{yeast,dry}}$ (g/l), was then calculated according to Equation :

$$C_{\text{yeast,dry}} = \frac{\left(m_{\text{crucible+yeast}} - m_{\text{crucible}}\right) \cdot \rho_{\text{wort}}}{\left(m_{\text{tube+sample}} - m_{\text{tube}}\right)} \tag{3}$$

where $m_{\text{crucible+yeast}}$ is the mass of the crucible containing the dried yeast (g)

m_{crucible} is the mass of the empty crucible (g)

ρ_{wort} is the density of the wort at the time of sampling (g/l)

$m_{\text{tube+sample}}$ is the mass of the centrifuge tube containing the sample (g)

m_{tube} is the mass of the empty centrifuge tube (g).

3.3.3 Yeast viability

The viability of a yeast sample was determined by first diluting the yeast into a 20% yeast slurry by adding the appropriate amount of MQ-H_2O. 10 µl of the slurry was then diluted further in a 2 ml Eppendorf tube by adding 990 µl of a 10 mM EDTA solution and mixing thoroughly with a vortex mixer (Vortex-Genie 2, Scientific Instruments Inc., USA). The cell number ($N_{\text{non-viable}}$) in this suspension (1:100 dilution) was counted with an integrated fluorescence microscope (NucleoCounter YC-100, ChemoMetec, Denmark). A further suspension was then made by mixing 50 µl of the 1:100 dilution with 450 µl of a lysis buffer (Reagent Y100, ChemoMetec, Denmark) in a 2 ml Eppendorf tube and mixing thoroughly with a

vortex mixer. The cell number (N_{total}) in this suspension (1:1000 dilution) was also counted with an integrated fluorescence microscope. The viability of the yeast sample, V (%), was then calculated using the following Equation (4):

$$V = 100 - \frac{N_{\text{non-viable}} \cdot d_{\text{non-viable}}}{N_{\text{total}} \cdot d_{\text{total}}} \cdot 100\% \quad (4)$$

where $N_{\text{non-viable}}$ is the number of counted cells in first suspension (no lysis buffer)

$d_{\text{non-viable}}$ is the dilution factor of the first suspension (i.e. 100)

N_{total} is the number of counted cells in the second suspension (lysis buffer)

d_{total} is the dilution factor of the second suspension (i.e. 1000).

3.3.4 Density, specific gravity, ethanol concentration and pH

The density, specific gravity, ethanol concentration and pH of samples was determined from the supernatant collected during yeast fresh mass analysis (section 3.3.1). The sample was poured into a 100 ml glass tube and the tube was capped with a glass stopper. The tubes were shaken vigorously for a few seconds at a time, after which pressure was released from the tubes, to degas the samples. This process was repeated at least five times for each sample. The decarbonated sample was then poured into a plastic vial and the vial was capped with a silicone cap. The vial was placed in a sample filling unit (Anton Paar Xsample 122, Anton Paar GmbH, Austria), and the density (Anton Paar Density Meter DMA 5000 M, Anton Paar GmbH, Austria), specific gravity, ethanol concentration (Anton Paar Alcolyzer Beer ME, Anton Paar GmbH, Austria) and pH (Anton Paar pH ME, Anton Paar GmbH, Austria) of the sample was measured.

3.3.5 Extract and attenuation

The apparent extract (AE; ° Plato) of the samples was estimated from the previously measured specific gravities (section 3.3.4) using the following approximation (Equation (5); Kobayashi *et al.*, 2005b):

$$AE = -202.414 \cdot SG^2 + 662.649 \cdot SG - 460.234 \quad (5)$$

where, SG is the specific gravity of the sample.

The apparent attenuation (AA; %) of the samples was estimated from the apparent and original extract (i.e. the apparent extract of the wort prior to pitching) using Equation (6):

$$AA = \left(1 - \frac{AE}{OE}\right) \cdot 100\% \quad (6)$$

where, AE is the apparent extract of the sample (° Plato)
OE is the original extract of the sample (° Plato).

The real extract (RE; ° Plato) of the samples was estimated from the apparent extract (AE; ° Plato) and the ethanol content (A_{ABW}; % (w/w)) using the following Equation (7) proposed by Hackbarth (2009):

$$\begin{aligned} RE = {} & 0.496815689 \cdot A_{ABW} + 1.001534136 \cdot AE - 0.000591051 \cdot A_{ABW} \cdot AE \\ & - 0.000294307 \cdot AE^2 - 0.0084747 \cdot A_{ABW}^2 + 0.000183564 \cdot A_{ABW}^3 \\ & + 0.000011151 \cdot AE^3 + 0.000002452 \cdot A_{ABW}^2 \cdot AE^2 \end{aligned} \quad (7)$$

where A_{ABW} is the ethanol content of the sample by weight (% (w/w))
AE is the apparent extract of the sample (° Plato).

The real attenuation (RA; %) of the samples was estimated from the real (RE) and original extract (OE) using Equation :

$$RA = \left(1 - \frac{RE}{OE}\right) \cdot 100\% \quad (8)$$

where, RE is the real extract of the sample (° Plato)

OE is the original extract of the sample (° Plato).

3.3.6 Vicinal diketone concentrations

The total concentration of vicinal diketones in samples was determined from the supernatant collected during yeast fresh mass analysis (section 3.3.1) according to Analytica-EBC method 9.10 (European Brewery Convention 2008). 15 ml of the supernatant was collected in a sterile vial, and frozen at -20° C until analysis. For analysis, the samples were first thawed and heated to 60° C, where they were kept for 90 minutes, in a headspace auto sampling unit (Headspace Autosampler 7000 HT, Tekmar-Dohrmann, USA) to convert any remaining α-acetolactate and α-acetohydroxybutyrate to diacetyl and 2,3-pentanedione, respectively. The samples were then analysed by headspace gas chromatography (HP 6890 Series GC System, Hewlett-Packard, USA; HP-5 50m · 320 µm · 1.05 µm column, Agilent, USA) with 2,3-hexanedione as an internal standard.

3.3.7 Amino acid concentrations

The concentrations of amino acids in samples were determined from the supernatant collected during yeast fresh mass analysis (section 3.3.1). 10 ml of the supernatant was collected in a sterile vial, and frozen at -20° C until analysis. For analysis, the samples were first thawed and the diluted to 1:40. 10 µl of the diluted sample was taken and mixed with 10 µl of norvaline (250 µM, internal standard) and 70 µl of boric acid buffer. The mixture was then vortexed for 30 seconds. Derivatization was done with AccQ•Fluor reagent kit. The AccQ•Fluor reagent was reconstituted with

acetonitrile (1 ml), and vortexed for 30 seconds. The mixture was heated to 55° C for 8 minutes, kept in an ultrasound bath for 5 minutes and finally vortexed for 60 seconds. The AccQ•Fluor reagent (10 µL) was added to the sample mixture, which was instantly vortexed for 60 seconds. Samples were kept at 5 °C before and during analysis.

Analysis was performed on an Acquity UPLC system (Waters Corporation, USA) with UV detector. Chromatography was performed using an Acquity Mass Trak™ (2.1 x 150 mm, 1.7 μm) column (Waters Corporation, USA), kept at 43 °C. Injection volume was 2.0 µL. Separation was performed using gradient elution with 10% (v/v) Amino Acid Analysis Concentrate A in water and Amino Acid Analysis Eluent B at a flow rate of 0.4 mL/min. The signal was detected at 260 nm (2.4 nm resolution, 20 points/second).

3.3.8 Aroma compound concentrations

The concentrations of various yeast-derived aroma compounds (acetaldehyde, alcohols and esters) in the samples were determined from the supernatant collected during yeast fresh mass analysis (section 3.3.1) by headspace-GC/MS. 10 ml of the supernatant was filtered (0.45 µm cellulose acetate filter) into a sterile vial, and frozen at -20° C until analysis. For analysis, the samples were first thawed and then incubated at 60 °C for 30 minutes. 1 ml of sample was then injected in the splitless injector (260 °C; flow 14.9 ml min^{-1}) of the gas chromatograph (Agilent 6890 Series; Palo Alto, CA, USA) combined with an MS detector (Agilent 5973 Network MSD, USA) and SPME autosampler (Combipal, Varian Inc., USA). Analytes were separated on a BPX5 capillary column of 60 m x 0.25 mm with phase thickness 1.0 µm (SGE Analytical Science Pty Ltd., Australia). Helium was used as carrier gas on constant flow mode 1.7 ml min^{-1}. The temperature program was started at 50 °C for 3 min, then 10 °C min^{-1} to 100 °C, followed by 5 °C min^{-1} to 140 °C and finally 15 °C min^{-1} to 260 °C, where the temperature was kept for 1 min. MSD was operated in electron-impact mode at 70 eV, in the full scan m/z 40-550. The ion source temperature was 230 °C and the interface was 280 °C. Compounds were identified

with retention times of corresponding standards and by comparing the mass spectra on Palisade Complete 600 K Mass Spectral Library (Palisade Mass Spectrometry, USA) and were quantitated with a standard curve. 1-Butanol was used as internal standard.

3.3.9 Fermentable sugar concentrations

The concentrations of fermentable sugars in samples were determined by high-performance anion exchange chromatography (HPAEC) (Dionex ICS-3000) with pulse amperometric detection (PAD) using CarboPac PA-1 analytical column (4 mm x 250 mm) and CarboPac PA-1 guard column (4 mm x 50 mm) at 30°C (Dionex Corp, USA). The system was equilibrated with 100 mM NaOH. 100 µL of a diluted sample was filtered (0.45 µm) and injected, after which 100 mM NaOH was run through the column (5 min). Separation was performed with a gradient (1 mL min^{-1}) of 100 mM to 300 mM NaOH in 3 minutes and then 300 mM NaOH to 250 mM NaOH + 75 mM Na-acetate in 15 minutes and washing was done with 100 mM NaOH + 300 mM Na-acetate and 300 mM NaOH. The flow rate was 1 mL min^{-1}. The results were confirmed by MSQ detection (HPAEC-MS) using a CarboPac PA200 (3mm × 250 mm) with a CarboPac PA200 guard (3 mm × 50 mm) column (Dionex) with a configuration as described by Bruggink *et al.* (2005) and a gradient as described by Mikkelson *et al.* (2013).

3.3.10 Foam stability test

The foam stability of bottled beer was tested using the NIBEM-T method (European Brewery Convention, 2008). The temperature of the beer was adjusted to 20° C in a water bath, and then transferred as foam to a standard glass via CO_2 overpressure (2 bar). The time taken for the foam to collapse 10 mm, 20 mm and 30 mm was measured using a NIBEM-T foam stability tester (Haffmans BV, Netherlands), according to the instructions in EBC-Analytica (European Brewery Convention, 2008).

4 Results

4.1 Effect of supplementing various amounts of valine to all-malt wort on the production of diacetyl during fermentation

4.1.1 Fermentation performance

The ethanol content (% v/v) and the estimated real extracts (weight %) of the worts used for the experimental fermentations of the first experiment as a function of fermentation time are presented in Figure 8. As can be seen from the plot, valine supplementation appears to have had no effect on either fermentation rate or final attenuation level. After fermentation had finished, all beers contained an ethanol content of around 6.5% (v/v). The yeast dry mass and fresh mass content of the worts (g/l) as a function of fermentation time is presented in Figure 9, and as can be seen from the plot, valine supplementation appears to have had no effect on the amount of yeast biomass produced during fermentation either, even though the amount of assimilable nitrogen available for yeast growth increases with the supplemented valine. The pH of the valine-supplemented worts remained similar throughout the fermentation as well (see Figure 1 in Appendix 3), suggesting that fermentation performance was not affected by the supplementation of various amounts of valine to the wort.

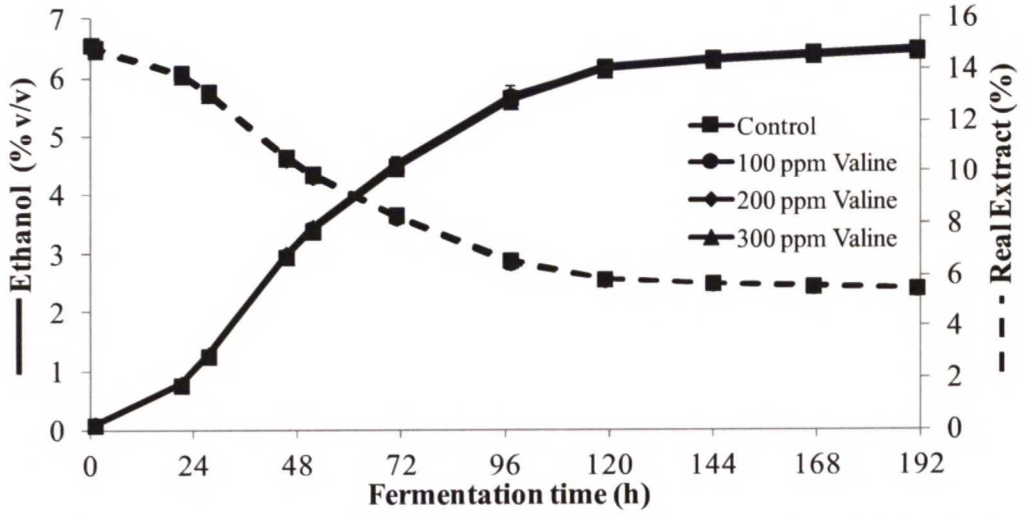

Figure 8 – The ethanol content (% v/v; solid line) and real extract (weight %; dashed line) of the valine-supplemented worts used for the experimental fermentations of the first experiment as a function of fermentation time (hours). Values are means from two independent fermentations. Error bars where visible represent standard deviation of mean.

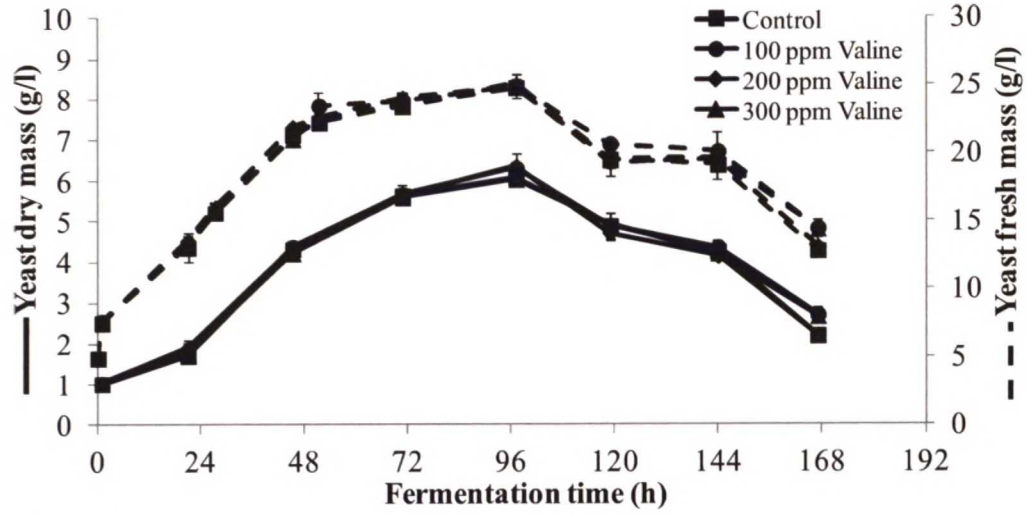

Figure 9 – The yeast dry mass content (g/l; solid line) and yeast fresh mass content (g/l; dashed line) of the valine-supplemented worts used for the experimental fermentations of the first experiment as a function of fermentation time (hours). Values are means from two independent fermentations. Error bars where visible represent standard deviation of mean.

4.1.2 VDK production

The concentrations of diacetyl and 2,3-pentanedione in the fermenting wort as a function of fermentation time are presented in Figure 10 and Figure 11 respectively. As can be seen from the plots, all amounts of valine supplementation to the all-malt wort lowered the maximum diacetyl concentration observed during fermentation, while it did not have as a significant impact on the production of 2,3-pentanedione. Increasing the amount of supplemented valine reduced the maximum concentration of diacetyl observed during fermentation for all supplementation levels. The diacetyl concentrations at the end of the fermentation (192 hours) were also lower for the valine-supplemented worts compared to the control worts, while the difference amongst the valine-supplemented worts was smaller compared to the difference observed at maximum diacetyl peak (around 48 to 96 hours into fermentation). The diacetyl reduction rate at the end of fermentation was similar for all worts, which can be explained by the fact that approximately the same amount of yeast biomass was available for reduction and the diacetyl peaks occurred at the same time during fermentation. The diacetyl concentrations of the beer at the end of fermentation were not reduced to levels under the flavour threshold (around 100 ppb in lager beers, but could be noticeably lower (see Section 2.3.2)) during the observed fermentation time period, however the diacetyl concentration (121.6 ppb) of the beer fermented from the wort supplemented with 300 ppm valine was closest to the threshold. If the diacetyl reduction rates remain similar after fermentation to what they were at the end of fermentation (around 1 ppb / hour for all worts), diacetyl concentrations would be decreased to below the flavour threshold 33 to 61 hours earlier in the beer fermented from the valine-supplemented worts compared to the control wort depending on valine supplementation amount. Diacetyl reduction rates would most likely decrease after fermentation though, as the amount of yeast in suspension decreases further. The 2,3-pentanedione concentrations remained under the flavour threshold of 900-1000 ppb for all the worts during the entire fermentation.

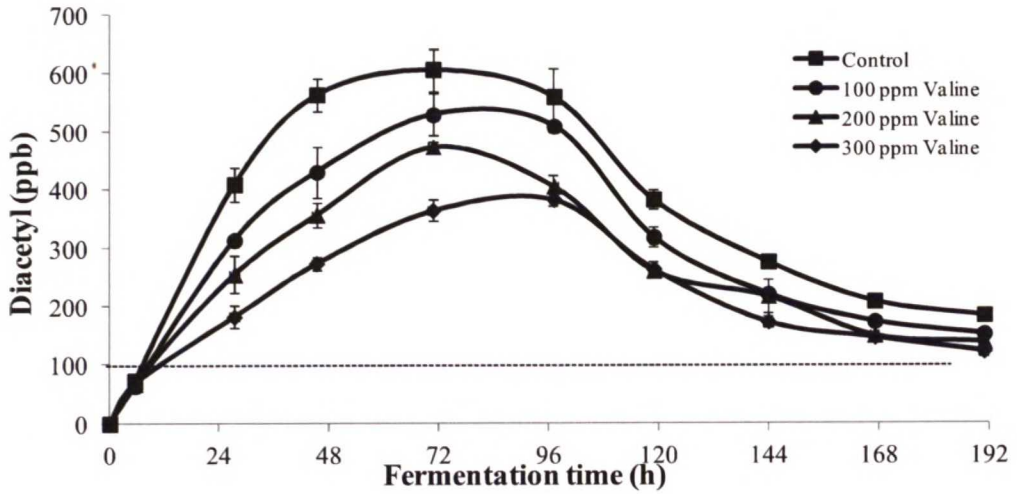

Figure 10 – The diacetyl concentration (ppb or µg/l) of the valine-supplemented worts used for the experimental fermentations of the first experiment as a function of fermentation time (hours). The dotted line at 100 ppb depicts the flavour threshold. Error bars where visible represent standard deviation of the sample.

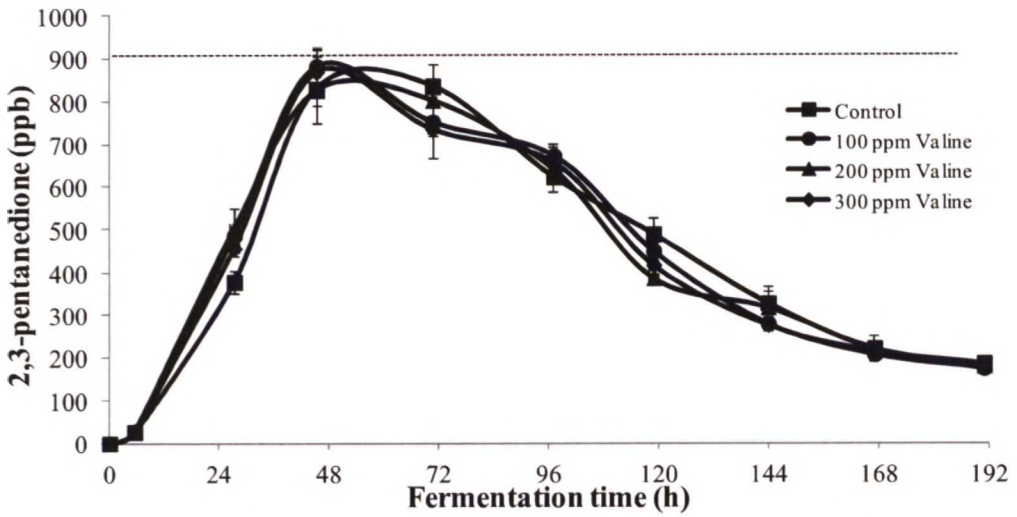

Figure 11 – The 2,3-pentanedione concentration (ppb or µg/l) of the valine-supplemented worts used for the experimental fermentations of the first experiment as a function of fermentation time (hours). The dotted line at 900 ppb depicts the flavour threshold. Values are means from two independent fermentations. Error bars where visible represent standard deviation of the sample.

4.2 Effects of valine supplementation and FAN content of wort on the production of diacetyl during fermentation

4.2.1 Fermentation performance

The ethanol content (% v/v) and the estimated real extracts (weight %) of the worts as a function of fermentation time is presented in Figure 12. As can be seen from the plot, the all-malt worts (Standard FAN) were attenuated slightly faster than the semi-synthetic worts (Reduced FAN), and valine supplementation appears to have had no effect on fermentation performance for either the all-malt wort or the semi-synthetic wort. After fermentation had finished, the beers fermented from all-malt worts (Standard FAN) contained an ethanol content of around 6.3% (v/v), while the beers fermented from the semi-synthetic worts (Reduced FAN) contained an ethanol content of around 6.9% (v/v) and also reached a higher attenuation level, since a higher ratio of its original extract was fermentable sugars (no non-fermentable sugars were added to the sugar solution). The higher ethanol content of the Reduced FAN fermentations can be explained by the fact that maltotriose was replaced by an equal amount of maltose in the sugar solution, although maltotriose is much less fermentable than maltose (Zheng *et al.*, 1994). During the lag phase (the first 30 hours), ethanol production rate was similar for all worts, but between approximately 30 and 48 hours after pitching, the ethanol production rate of the all-malt worts (Standard FAN) was higher than the semi-synthetic worts (Reduced FAN). The pH of the valine-supplemented worts remained similar to their respective control worts throughout the fermentation (see Figure 2 in Appendix 3), while the pH of the semi-synthetic worts during fermentation was lower than that of the all-malt worts, most likely due to loss of buffer capacity through dilution.

The yeast dry mass and fresh mass content (g/l) of the worts as a function of fermentation time is presented in Figure 13, and as can be seen from the plot, the amount of yeast biomass in the all-malt worts (Standard FAN) was at times over 20% higher than the amount of yeast biomass in the semi-synthetic worts (Reduced FAN). Valine supplementation appears to have had no effect on the amount of yeast biomass produced during fermentation either. The higher yeast biomass content of

the all-malt worts can be explained by the higher FAN content of the worts, as more assimilable nitrogen is available for yeast growth, and the higher yeast biomass content can in turn explain the slightly faster fermentation rate of the all-malt worts. The decrease in yeast biomass towards the end of fermentation can be explained by an increased amount of sedimented yeast in the fermentation vessels towards the end of fermentation.

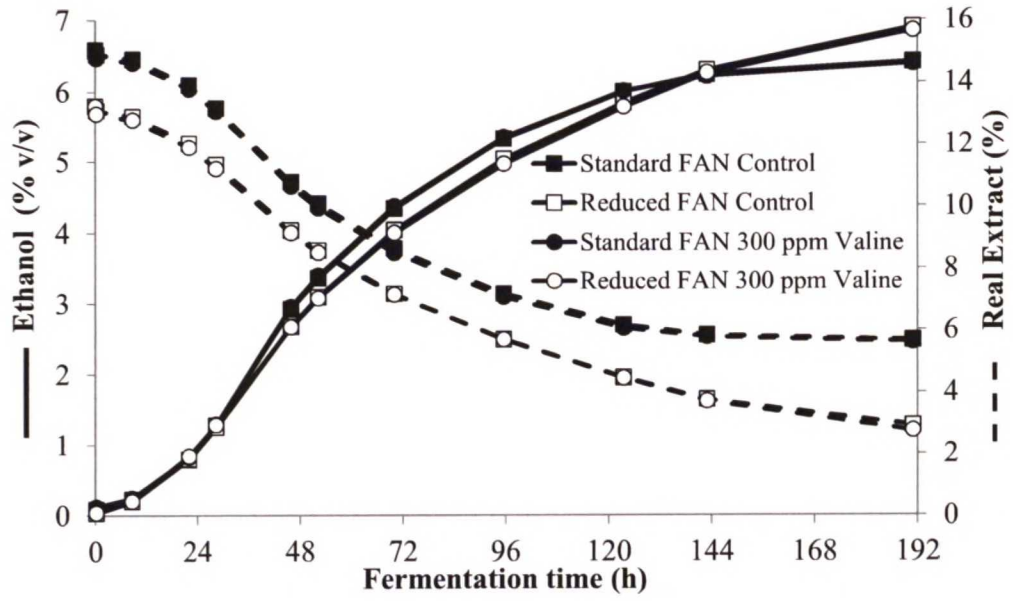

Figure 12 – The ethanol content (% v/v; solid line) and real extract (weight %; dashed line) of the valine-supplemented all-malt and semi-synthetic worts used for the experimental fermentations of the second experiment as a function of fermentation time (hours). Values are means from two independent fermentations. Error bars where visible represent standard deviation of mean.

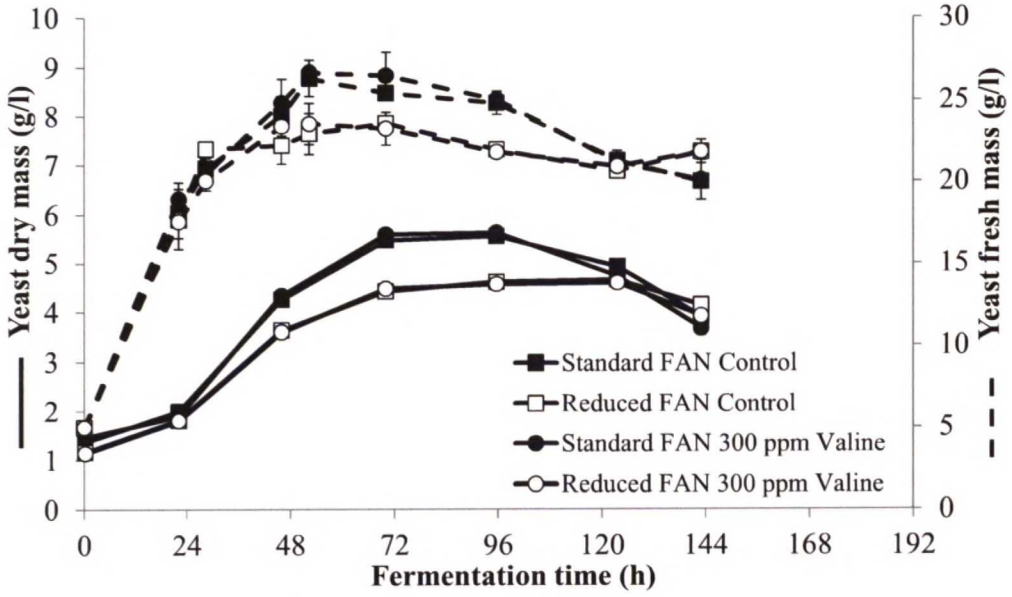

Figure 13 – The yeast dry mass content (g/l; solid line) and yeast fresh mass content (g/l; dashed line) of the valine-supplemented all-malt and semi-synthetic worts used for the experimental fermentations of the second experiment as a function of fermentation time (hours). Values are means from two independent fermentations. Error bars where visible represent standard deviation of mean.

4.2.2 VDK production

The concentrations of diacetyl and 2,3-pentanedione in the fermenting wort as a function of fermentation time are presented in Figure 14 and Figure 16 (Standard FAN) and Figure 15 and Figure 17 (Reduced FAN) respectively. As can be seen from the plots, valine supplementation to both the all-malt wort (Standard FAN) and the semi-synthetic wort (Reduced FAN) significantly lowered the maximum diacetyl concentration observed during fermentation, while it did not have as large of an impact on the production of 2,3-pentanedione. The diacetyl concentrations at the end of the fermentation (143 hours for Standard FAN worts and 191 hours for Reduced FAN worts) were also lower for both the valine-supplemented worts compared to their respective control worts. The diacetyl reduction rate at the end of fermentation was greater in the semi-synthetic wort fermentations, which can be explained by the fact that more biomass was in suspension and available for reduction and the diacetyl peak was at a later time of the fermentation for the semi-synthetic worts. The broader and later diacetyl peak of the semi-synthetic worts compared to the all-malt worts can be explained by the similar trend of a broader and later biomass peak in these fermentations (see yeast dry mass content in Figure 13).

The valine-supplemented semi-synthetic wort (Reduced FAN 300 ppm Valine) displayed the lowest diacetyl concentrations during peak fermentation (around 30 to 96 hours). The semi-synthetic control wort during the same time period also showed lower diacetyl concentrations than the all-malt control wort. The diacetyl concentrations of the worts were not reduced to levels under the flavour threshold (around 100 ppb in lager beers, but could be even lower than 100 ppb (see Section 2.3.2)) during the observed fermentation time period. The 2,3-pentanedione concentrations remained under the flavour threshold of 900-1000 ppb for all the worts during the entire fermentation.

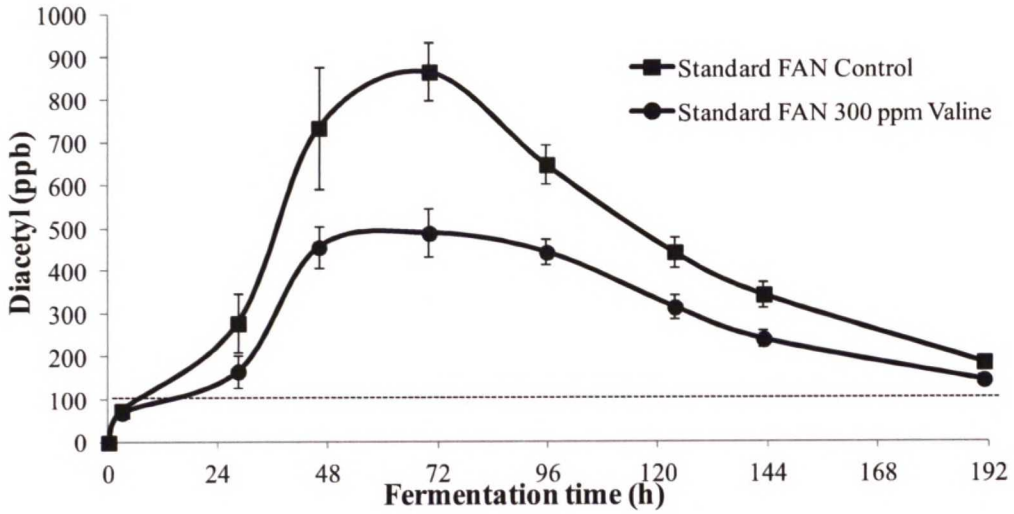

Figure 14 – The diacetyl concentration (ppb or µg/l) of the all-malt worts used for the experimental fermentations of the second experiment as a function of fermentation time (hours). The dotted line at 100 ppb depicts the flavour threshold. Values are means from two independent fermentations. Error bars where visible represent standard deviation of mean.

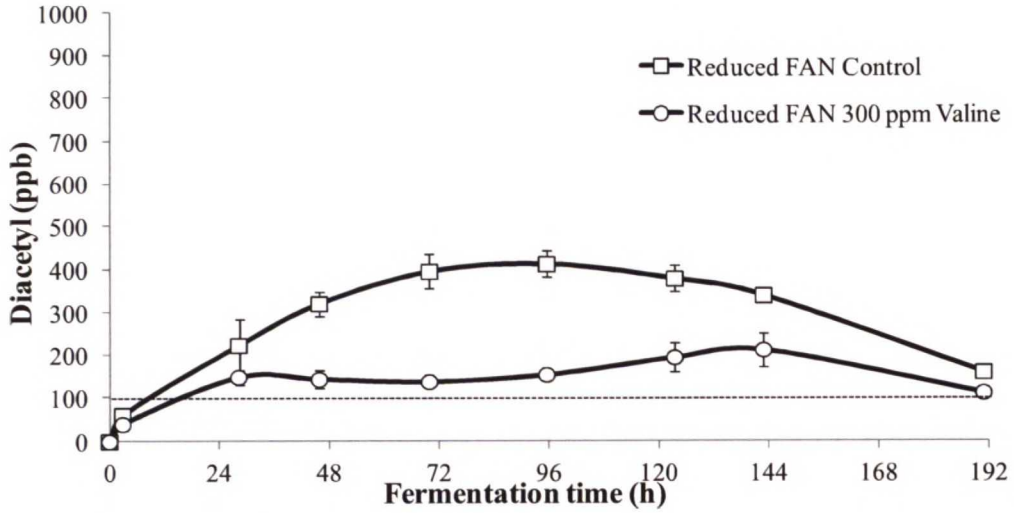

Figure 15 – The diacetyl concentration (ppb or µg/l) of the semi-synthetic worts used for the experimental fermentations of the second experiment as a function of fermentation time (hours). The dotted line at 100 ppb depicts the flavour threshold. Values are means from two independent fermentations. Error bars where visible represent standard deviation of mean.

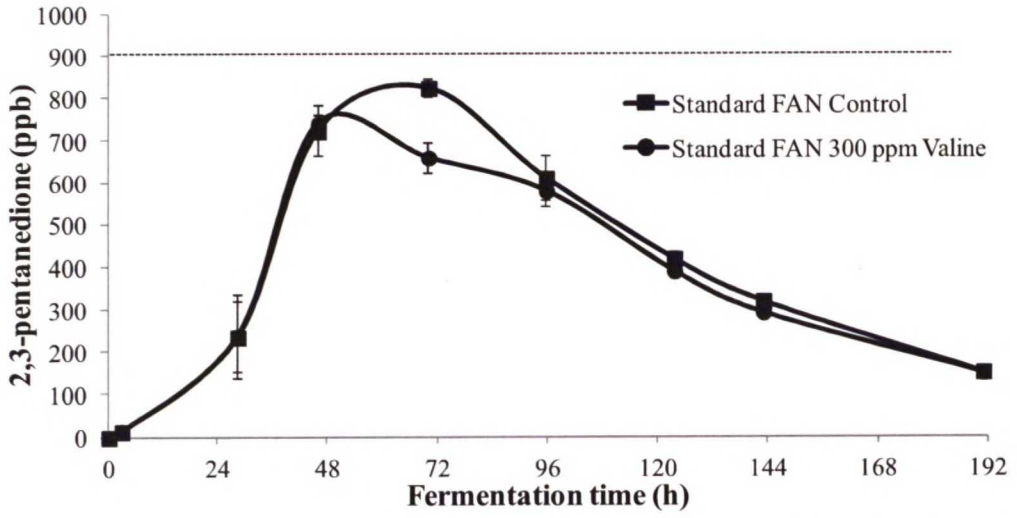

Figure 16 – The 2,3-pentanedione concentration (ppb or µg/l) of the all-malt worts used for the experimental fermentations of the second experiment as a function of fermentation time (hours). The dotted line at 900 ppb depicts the flavour threshold. Values are means from two independent fermentations. Error bars where visible represent standard deviation of mean.

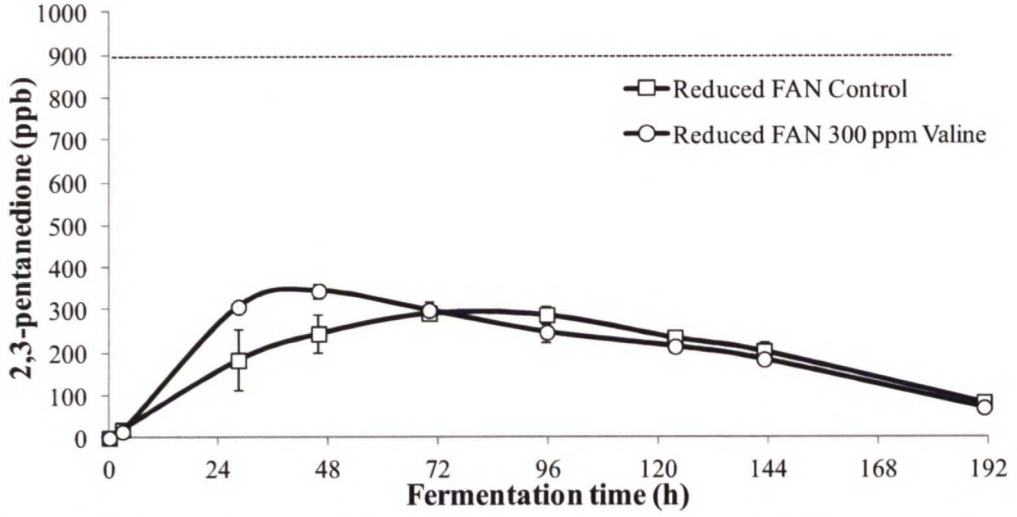

Figure 17 – The 2,3-pentanedione concentration (ppb or µg/l) of the semi-synthetic worts used for the experimental fermentations of the second experiment as a function of fermentation time (hours). The dotted line at 900 ppb depicts the flavour threshold. Values are means from two independent fermentations. Error bars where visible represent standard deviation of mean.

4.2.3 Amino acid uptake

The concentrations of valine, isoleucine and leucine in the fermenting all-malt wort (Standard FAN) as a function of fermentation time are presented in Figure 18, Figure 19, and Figure 20, respectively. As can be seen from the plots, the valine uptake rate of the yeast increased when valine was supplemented to the wort, while the uptake rate of isoleucine and leucine was not as affected and only slightly decreased in the first 24 hours of fermentation. The difference between the initial valine concentration and the valine concentration at the end of fermentation (191 hours) was also greater in the valine-supplemented wort compared to the control wort. A combined plot of the change in valine concentration (i.e. valine uptake rate; ppm/h) and the change in diacetyl concentration (i.e. the diacetyl production rate; ppb/h) of the fermenting all-malt wort (Standard FAN) as a function of fermentation time is presented in Figure 21. As can be seen from the plot, the valine uptake rate of the yeast during the first three days of fermentation is higher (i.e. the change in valine concentration is more negative) in the valine-supplemented wort compared to the control wort. Concurrently, the diacetyl production rate of the yeast is lower in the valine-supplemented wort compared to the control wort, suggesting that valine uptake rate negatively correlates with the amount of diacetyl produced during the growth phase of fermentation.

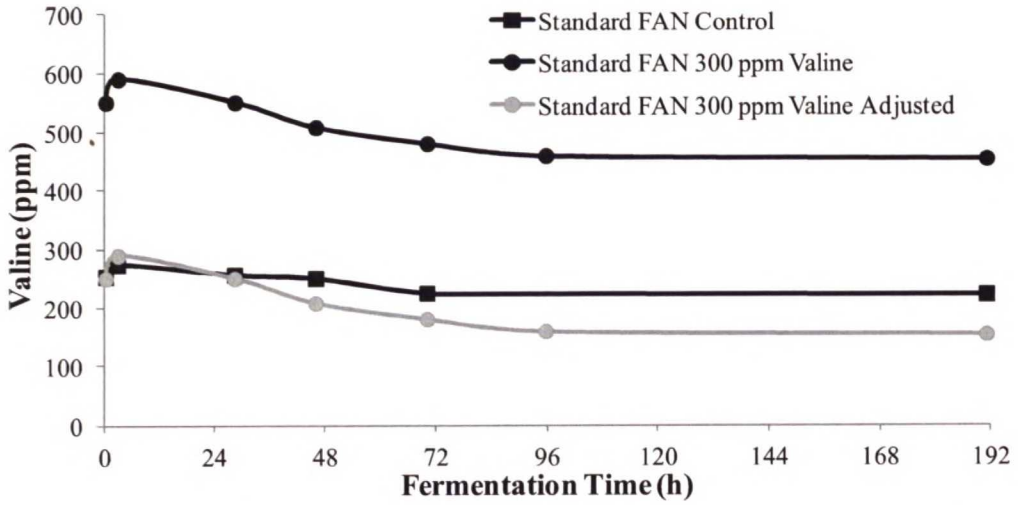

Figure 18 – The valine concentration (ppm or mg/l) of the all-malt worts used for the experimental fermentations of the second experiment as a function of fermentation time (hours). The adjusted plot contains the values of the 'Standard FAN 300 ppm Valine' plot with 300 ppm subtracted, to facilitate comparison between the slopes. The relative standard deviation of the amino acid concentrations was below 2.2% for all measurements.

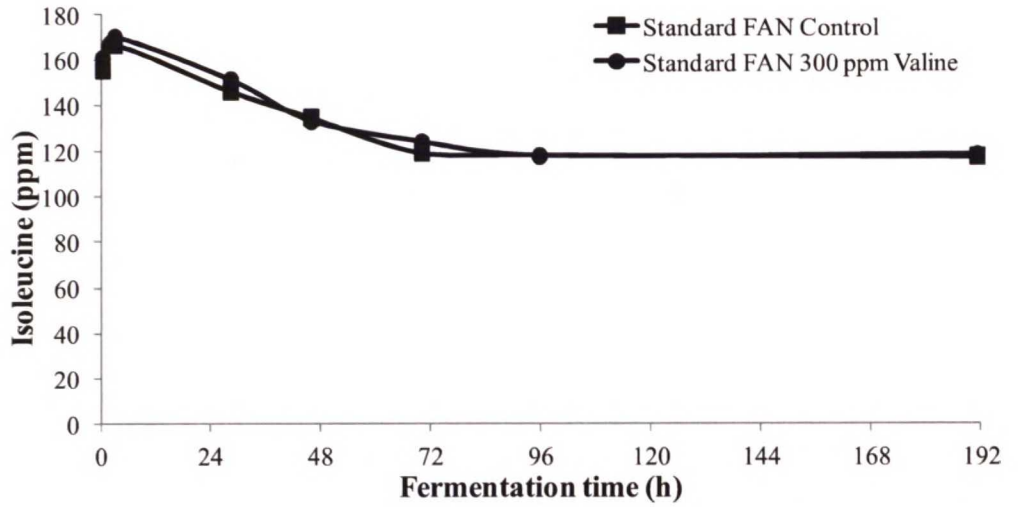

Figure 19 – The isoleucine concentration (ppm or mg/l) of the all-malt worts used for the experimental fermentations of the second experiment as a function of fermentation time (hours). The relative standard deviation of the amino acid concentrations was below 2.2% for all measurements.

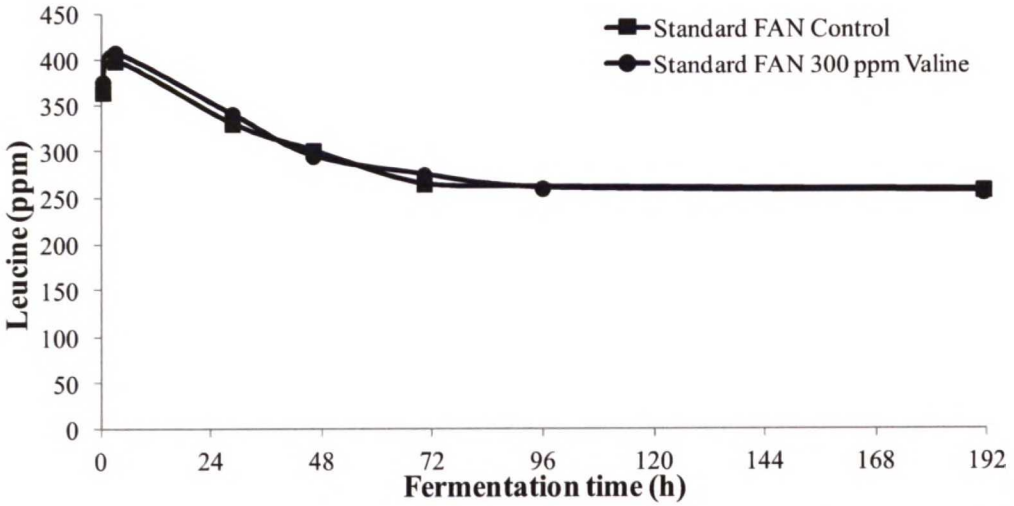

Figure 20 – The leucine concentration (ppm or mg/l) of the all-malt worts used for the experimental fermentations of the second experiment as a function of fermentation time (hours). The relative standard deviation of the amino acid concentrations was below 2.2% for all measurements.

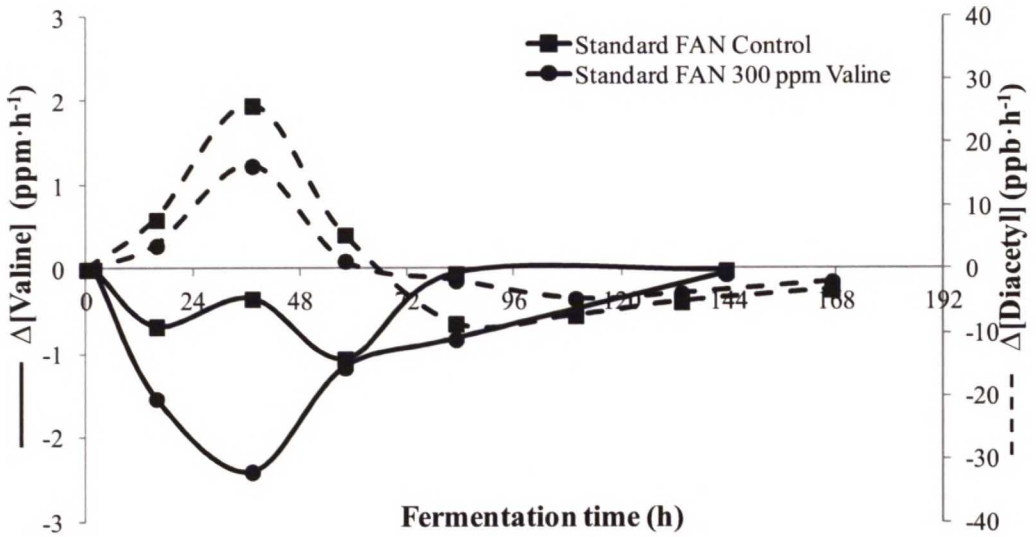

Figure 21 – The average linear change in wort valine concentration (ppm/h) and diacetyl concentration (ppb/h) of the all-malt worts used for the experimental fermentations of the second experiment as a function of fermentation time (hours).

4.3 Effect of altering the amino acid spectrum of wort on the production of diacetyl during fermentation

4.3.1 Fermentation performance

The ethanol content (% v/v) and the estimated real extracts (weight %) of the worts used for the first experimental fermentations of the third experiment as a function of fermentation time are presented in Figure 22 (Standard FAN) and Figure 23 (Reduced FAN). As can be seen from the plots, there was not much of a difference in fermentation rate between the different fermentations, and the all-malt worts (Standard FAN) were attenuated just slightly faster than the semi-synthetic worts (Reduced FAN), while amino acid supplementation appears to have had no effect on fermentation performance for either the all-malt wort or the semi-synthetic wort. After fermentation had finished, the beers fermented from all-malt worts (Standard FAN) contained an ethanol content of around 6.13% (v/v), while the beers fermented from the semi-synthetic worts (Reduced FAN) contained an ethanol content of around 6.17% (v/v) and again also reached a higher attenuation level, since a higher ratio of its original extract was fermentable sugars (no non-fermentable sugars were added to the sugar solution). The slightly lower ethanol content of the beers from this experiment compared to the first experiment can be explained by the fact that the original extract of the wort in the second experiment was slightly lower, due to the addition of the amino acid and control solutions. This also explains the small differences in real extract among similar worts (portrayed in Figure 22 and Figure 23) even though ethanol content was similar.

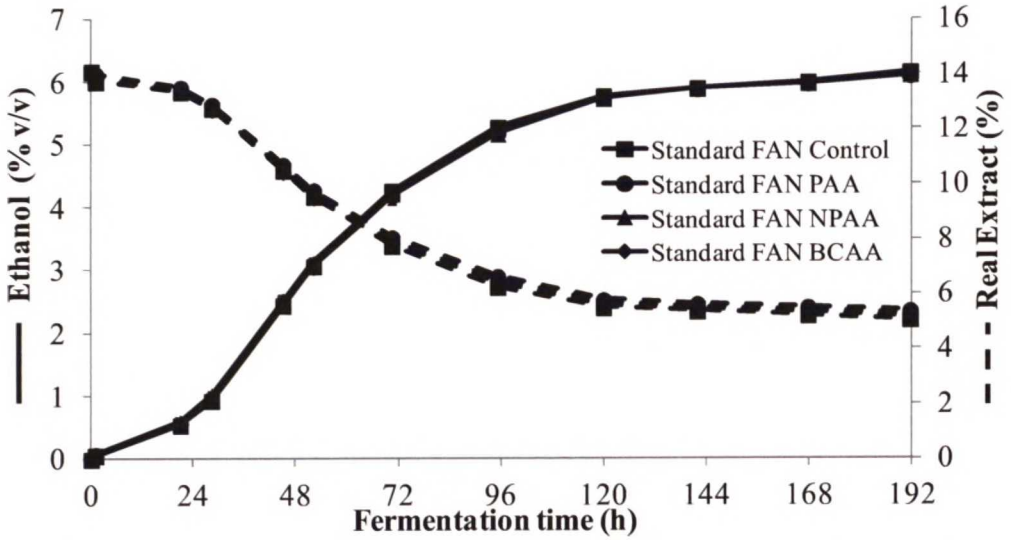

Figure 22 – The ethanol content (% v/v; solid line) and real extract (weight %; dashed line) of the all-malt worts used for the first experimental fermentations of the third experiment as a function of fermentation time (hours). Values are means from two independent fermentations. Error bars where visible represent standard deviation of mean.

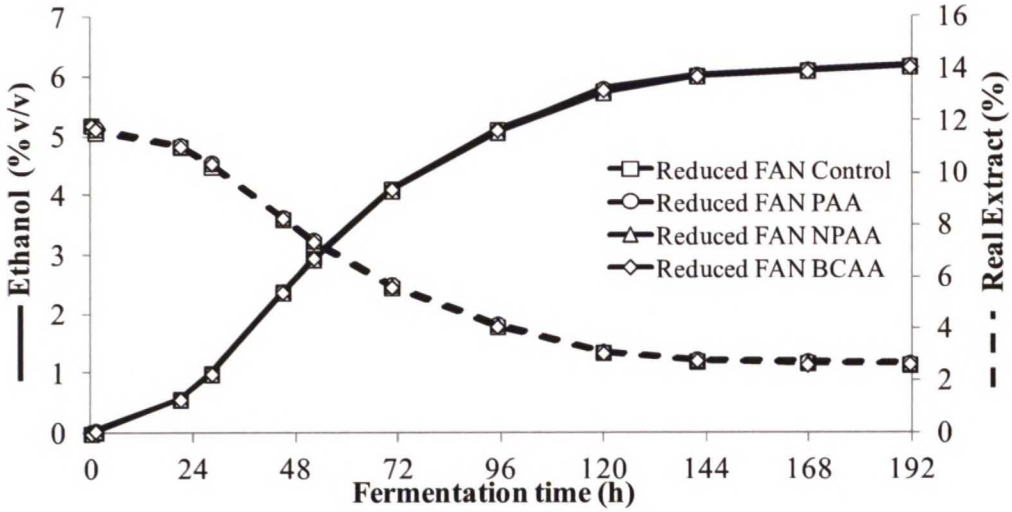

Figure 23 – The ethanol content (% v/v; solid line) and real extract (weight %; dashed line) of the semi-synthetic worts used for the first experimental fermentations of the third experiment as a function of fermentation time (hours). Values are means from two independent fermentations. Error bars where visible represent standard deviation of mean.

The yeast dry mass and fresh mass content (g/l) of the worts as a function of fermentation time are presented in Figure 24 (Standard FAN) and Figure 25 (Reduced FAN). The amount of yeast biomass in the worts was quite similar, but the all-malt worts (Standard FAN) had a slightly higher amount (around 15%) of yeast biomass compared to the semi-synthetic worts (Reduced FAN) between 48 and 120 hours after pitch. Amino acid supplementation appears to have had no effect on the amount of yeast biomass produced during fermentation either. The higher yeast biomass content of the all-malt worts can be explained by the higher FAN content of the worts, as more assimilable nitrogen is available for yeast growth. The higher biomass content of the all-malt worts during 48 to 120 hours after pitch can also explain the slightly faster fermentation rate of these worts during that time period. The decrease in yeast biomass towards the end of fermentation can be explained by an increased amount of sedimented yeast in the fermentation vessels towards the end of fermentation.

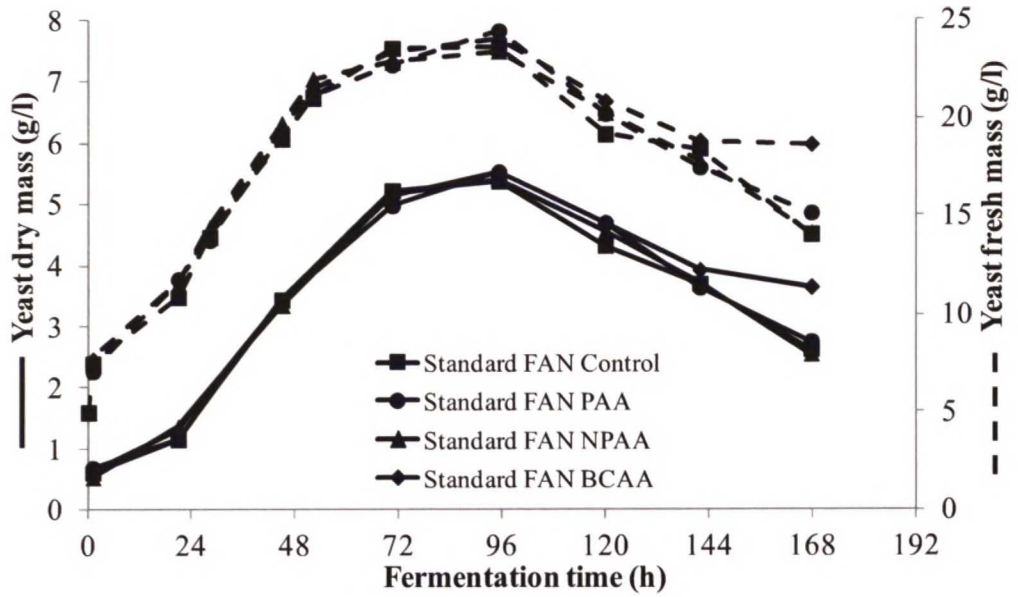

Figure 24 – The yeast dry mass content (g/l; solid line) and yeast fresh mass content (g/l; dashed line) of the all-malt worts (Standard FAN) used for the first experimental fermentations of the third experiment as a function of fermentation time (hours). Values are means from two independent fermentations. Error bars where visible represent standard deviation of mean.

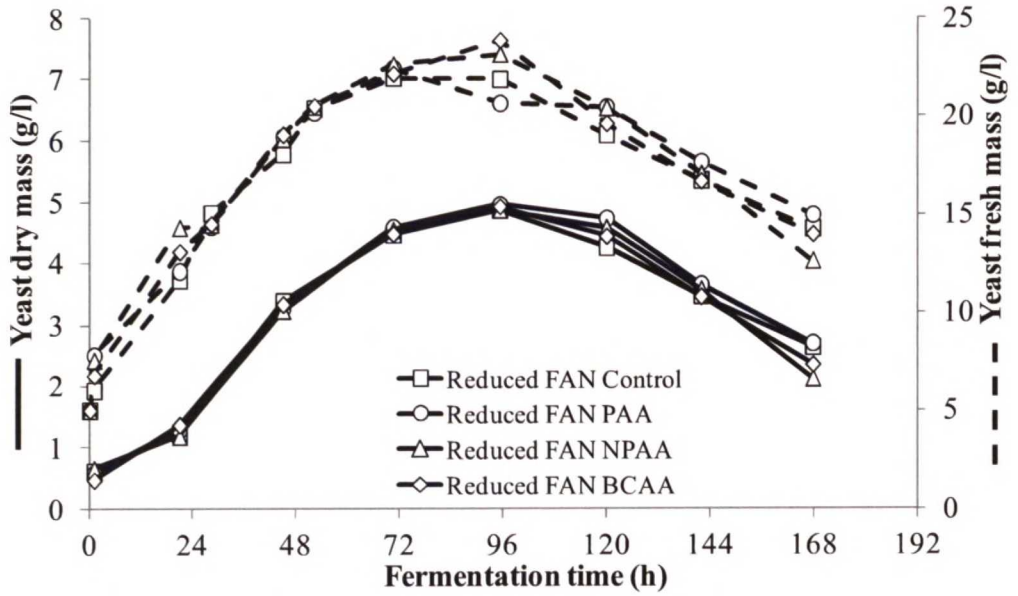

Figure 25 – The yeast dry mass content (g/l; solid line) and yeast fresh mass content (g/l; dashed line) of the semi-synthetic worts (Reduced FAN) used for the first experimental fermentations of the third experiment as a function of fermentation time (hours). Values are means from two independent fermentations. Error bars where visible represent standard deviation of mean.

The pH of the worts as a function of fermentation time is presented in Figure 26 (Standard FAN) and Figure 27 (Reduced FAN). As can be seen from the plots, the pH change of the worts remained quite similar throughout the fermentation, with the exception of the worts supplemented with the PAA solution, which raised the pH of the wort quite considerably compared to the control, NPAA and BCAA worts. This rise in pH is caused by the relatively high concentrations of lysine and arginine in the PAA solution, which make the solution alkaline. The pH of the semi-synthetic worts during fermentation was again lower than that of the all-malt worts, most likely due to loss of buffer capacity through dilution.

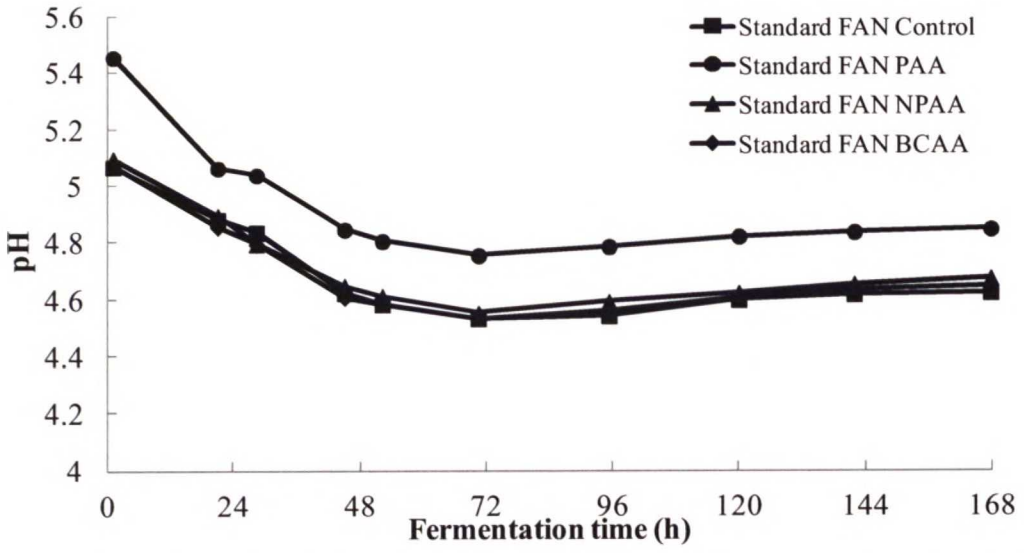

Figure 26 – The pH of the all-malt worts (Standard FAN) used for the first experimental fermentations of the third experiment as a function of fermentation time (hours). Values are means from two independent fermentations. Error bars where visible represent standard deviation of mean.

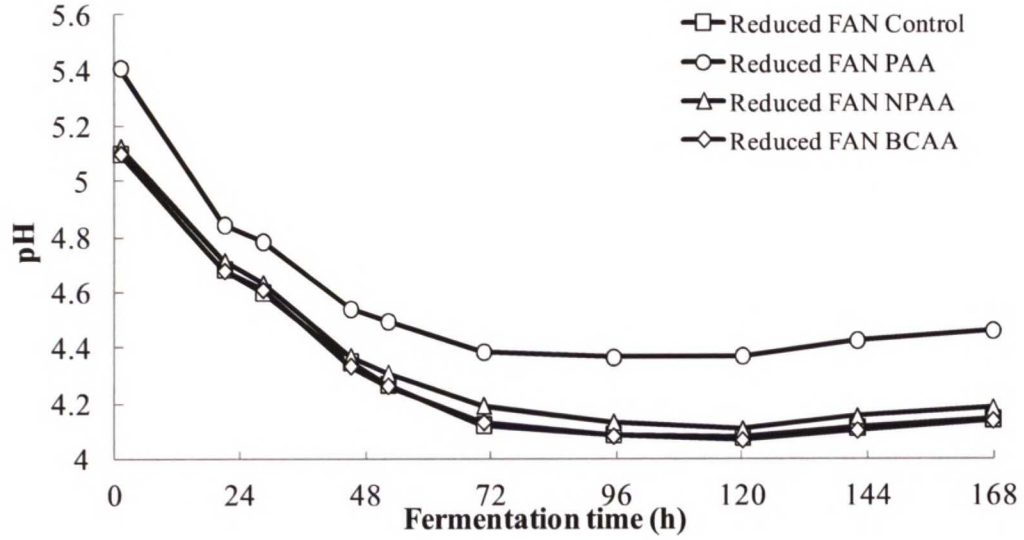

Figure 27 – The pH of the semi-synthetic worts (Reduced FAN) used for the first experimental fermentations of the third experiment as a function of fermentation time (hours). Values are means from two independent fermentations. Error bars where visible represent standard deviation of mean.

The ethanol content (% v/v) and the estimated real extracts (weight %) of the worts used for the second experimental fermentations (fermentations were repeated, since the pH of the PAA-supplemented worts were considerably higher than the control worts) of the third experiment as a function of fermentation time are presented in Figure 28. As can be seen from the plots, there was again not much of a difference in fermentation rate between the different fermentations, with the all-malt worts (Standard FAN) attenuating slightly faster than the semi-synthetic worts (Reduced FAN), while amino acid supplementation appears to have had no effect on fermentation performance for either the all-malt wort or the semi-synthetic wort. After fermentation had finished, the beers fermented from all-malt worts (Standard FAN) contained an ethanol content of around 6.25% (v/v), while the beers fermented from the semi-synthetic worts (Reduced FAN) contained an ethanol content of around 6.15% (v/v). Hence, the yeast attenuated the all-malt wort slightly more during the second fermentations compared to the first fermentations.

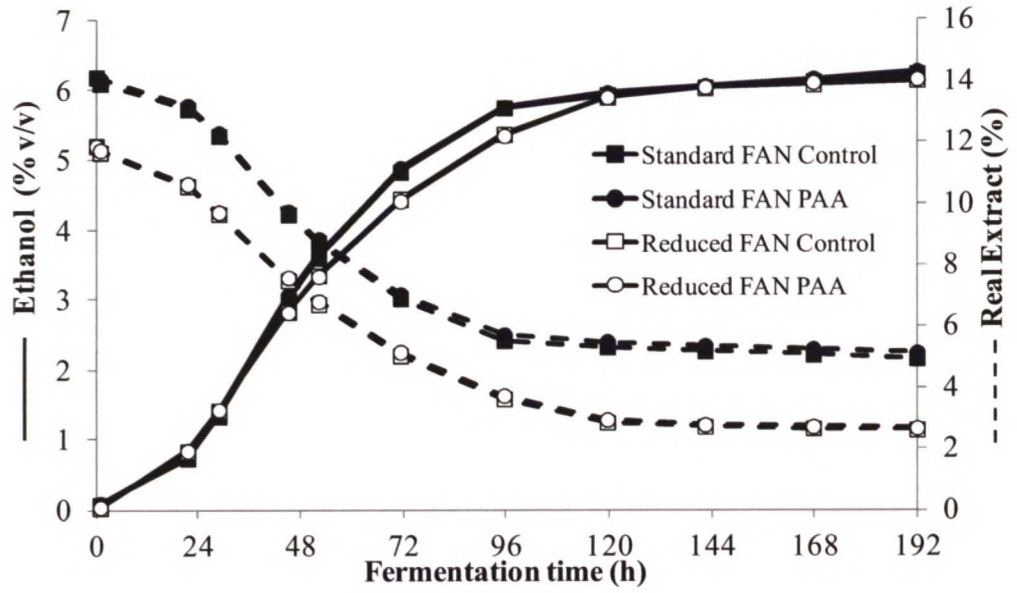

Figure 28 – The ethanol content (% v/v; solid line) and real extract (weight %; dashed line) of the worts used for the second experimental fermentations of the third experiment as a function of fermentation time (hours). Values are means from two independent fermentations. Error bars where visible represent standard deviation of mean.

The yeast dry mass and fresh mass content (g/l) of the worts used for the second experimental fermentations of the third experiment as a function of fermentation time are presented in Figure 29, and as can be seen from the plot, the amount of yeast biomass in the worts was quite similar, but the all-malt worts (Standard FAN) had a slightly higher amount (around 15%) of yeast biomass compared to the semi-synthetic worts (Reduced FAN) from 28 hours after pitch until the end of the fermentation. Again, the supplementation of the PAA solution appears to have had no effect on the amount of yeast biomass produced during fermentation. Compared to the first experimental fermentations, the second experimental fermentations produced slightly higher amounts of yeast biomass, which can explain the slightly increased fermentation rate of the fermentations as well. In addition, the wort used for the second experimental fermentations contained more trub (i.e. protein precipitate and other solid matter), because it was the final wort in the keg, which especially in the beginning of the fermentation most likely affected the measured yeast biomass values, as the trub was in suspension.

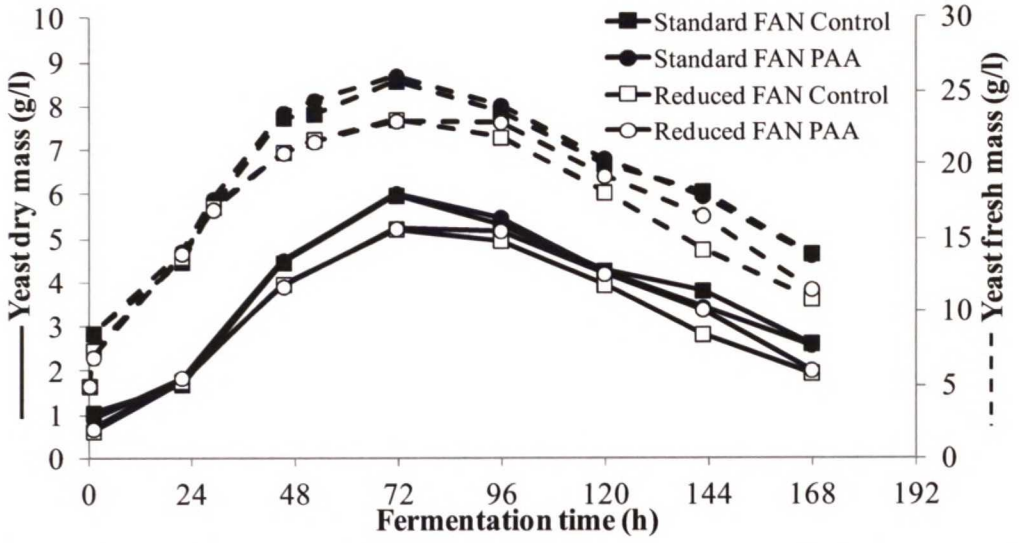

Figure 29 – The yeast dry mass content (g/l; solid line) and yeast fresh mass content (g/l; dashed line) of worts used for the second experimental fermentations of the third experiment as a function of fermentation time (hours). Values are means from two independent fermentations. Error bars where visible represent standard deviation of mean.

The pH of the worts as a function of fermentation time is presented in Figure 30 (Standard FAN) and Figure 31 (Reduced FAN). As can be seen from the plots, the addition of lactic acid to the PAA-supplemented worts reduced the pH difference compared to the control worts in the beginning of the fermentation, but the difference increased when the fermentation rate increased after about 28 hours after pitch. The pH difference between the PAA-supplemented and control worts was still much lower than during the first experimental fermentations, meaning it should have less of an effect on the concentration of VDK in the wort.

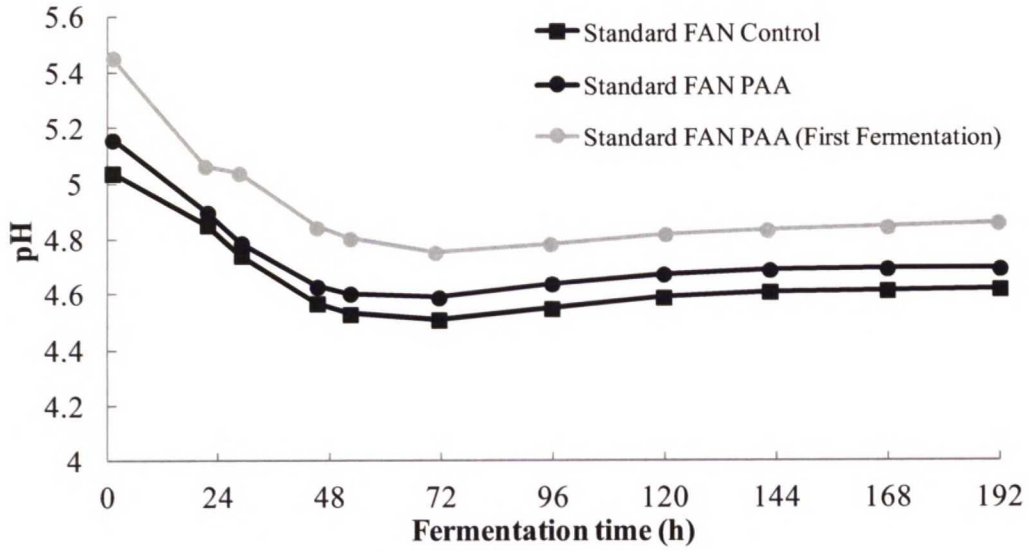

Figure 30 – The pH of the all-malt worts (Standard FAN) used for the second experimental fermentations of the third experiment as a function of fermentation time (hours). The grey-coloured curve represents the pH of the PAA-supplemented all-malt wort (Standard FAN PAA) during the first experimental fermentations. Values are means from two independent fermentations. Error bars where visible represent standard deviation of mean.

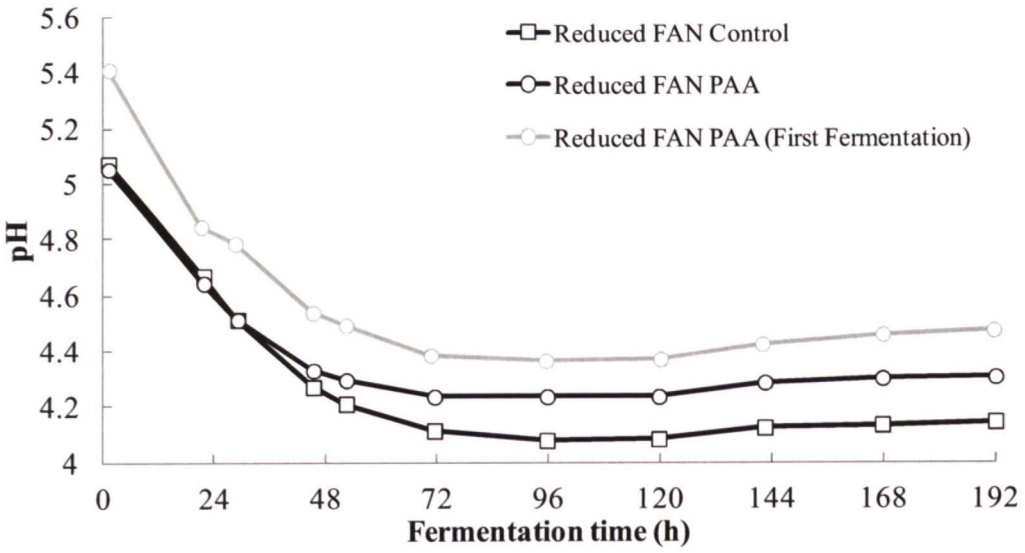

Figure 31 – The pH of the semi-synthetic worts (Reduced FAN) used for the second experimental fermentations of the third experiment as a function of fermentation time (hours). The grey-coloured curve represents the pH of the PAA-supplemented semi-synthetic wort (Reduced FAN PAA) during the first experimental fermentations. Values are means from two independent fermentations. Error bars where visible represent standard deviation of mean.

4.3.2 VDK production

The concentrations of diacetyl and 2,3-pentanedione in the fermenting worts used for the first experimental fermentations of the third experiment as a function of fermentation time are presented in Figure 32 and Figure 34 (Standard FAN) and Figure 33 and Figure 35 (Reduced FAN) respectively. The diacetyl concentrations of the PAA-supplemented worts of the first experimental fermentations were not measured, as the pH of the worts was significantly higher than that of the control worts. As can be seen from the plots, compared to the control worts, supplementation of the NPAA solution increased the maximum diacetyl concentration observed during fermentation of both the all-malt wort (Standard FAN) and the semi-synthetic wort (Reduced FAN), while supplementation of the BCAA solution slightly decreased the maximum diacetyl concentration observed during fermentation of the Standard FAN wort, while it increased the maximum diacetyl concentration observed during fermentation of the Reduced FAN wort. Supplementation of both the NPAA and BCAA solution lowered the maximum 2,3-pentanedione concentration observed during fermentation. Despite having an increased maximum diacetyl concentration during fermentation, the BCAA-supplemented Reduced FAN wort had slightly lower concentrations of diacetyl at the end of fermentation (191 hours) compared to the control wort, as the diacetyl peak occurred earlier during fermentation. The diacetyl concentrations at the end of the fermentation (191 hours) were the highest for the NPAA-supplemented wort and lowest for the BCAA-supplemented wort with both wort types. Again the diacetyl reduction rate at the end of fermentation was larger in the all-malt wort fermentations, which can be explained by the fact that more biomass was available for reduction.

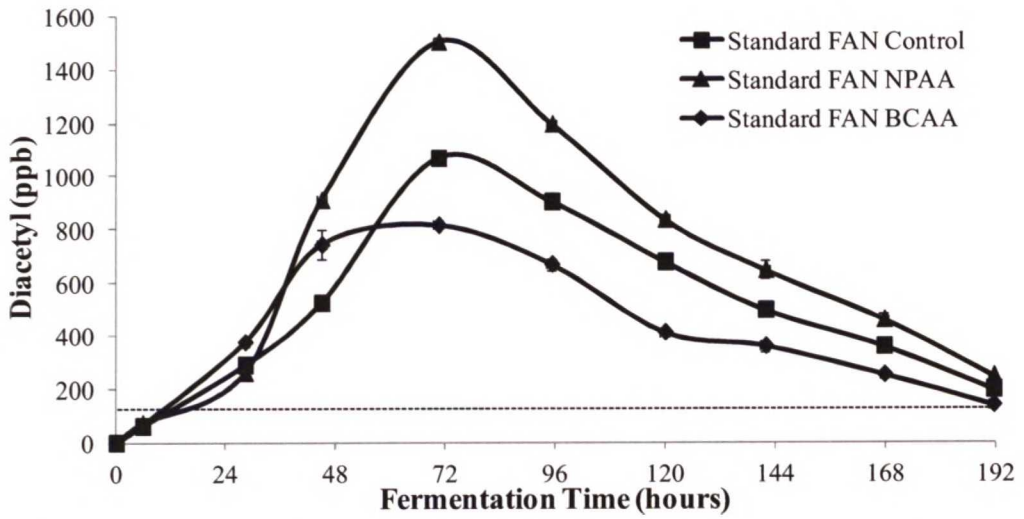

Figure 32 – The diacetyl concentration (ppb or µg/l) of the all-malt worts (Standard FAN) used for the first experimental fermentations of the third experiment as a function of fermentation time (hours). The dotted line at 100 ppb depicts the flavour threshold. Error bars where visible represent standard deviation of sample.

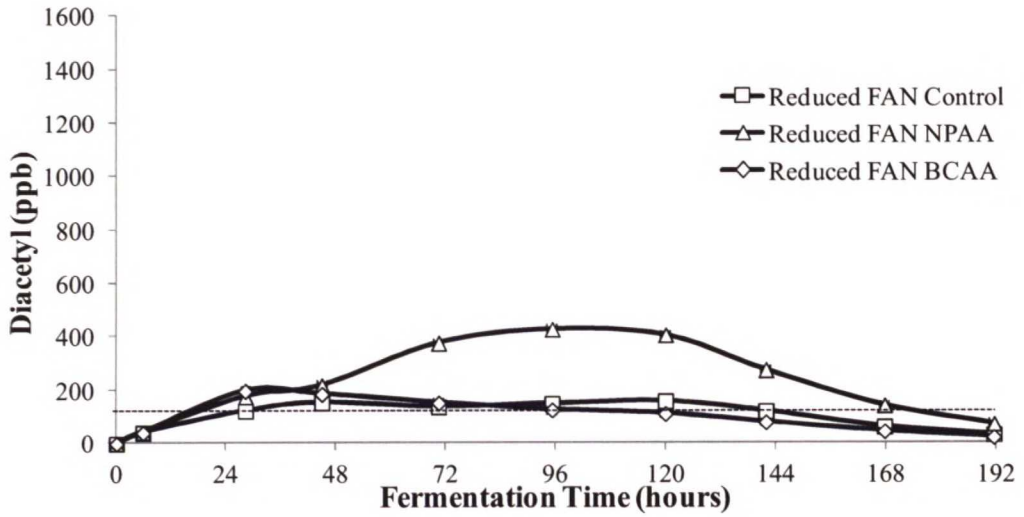

Figure 33 – The diacetyl concentration (ppb or µg/l) of the semi-synthetic worts (Reduced FAN) used for the second experimental fermentations of the second experiment as a function of fermentation time (hours). The dotted line at 100 ppb depicts the flavour threshold. Error bars where visible represent standard deviation of sample.

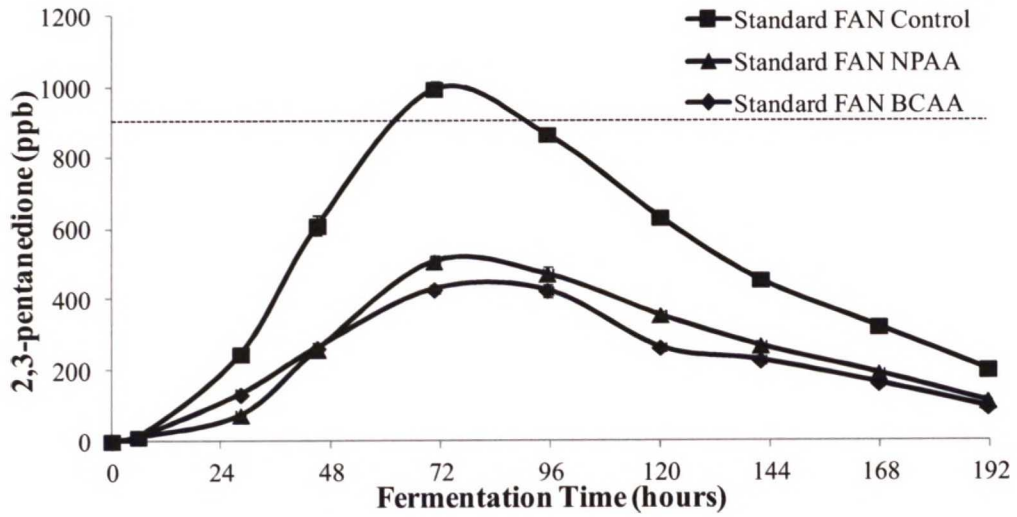

Figure 34 – The 2,3-pentanedione concentration (ppb or µg/l) of the all-malt worts (Standard FAN) used for the first experimental fermentations of the third experiment as a function of fermentation time (hours). The dotted line at 900 ppb depicts the flavour threshold. Error bars where visible represent standard deviation of sample.

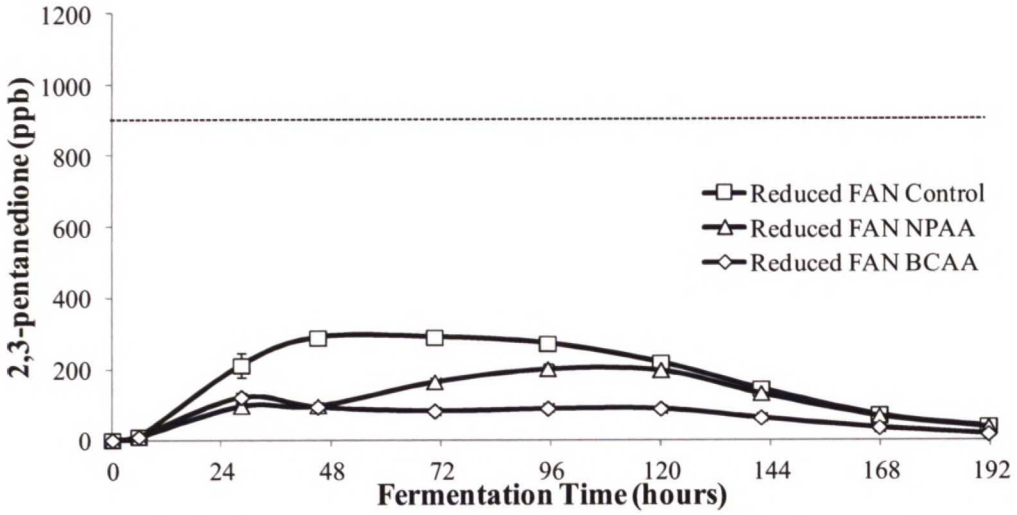

Figure 35 – The 2,3-pentanedione concentration (ppb or µg/l) of the semi-synthetic worts (Reduced FAN) used for the first experimental fermentations of the third experiment as a function of fermentation time (hours). The dotted line at 900 ppb depicts the flavour threshold. Error bars where visible represent standard deviation of sample.

The concentrations of diacetyl and 2,3-pentanedione in the fermenting worts used for the second experimental fermentations of the third experiment as a function of fermentation time are presented in Figure 36 and Figure 38 (Standard FAN) and Figure 37 and Figure 39 (Reduced FAN) respectively. Supplementation of the PAA solution increased the maximum diacetyl concentration observed during fermentation slightly in the all-malt wort (Standard FAN) and significantly in the semi-synthetic wort (Reduced FAN). The maximum 2,3-pentanedione concentrations observed during fermentation showed similar trends to the diacetyl concentration, with increases in both wort types as a result of PAA supplementation. Towards the end of fermentation, the difference in diacetyl concentrations of the PAA-supplemented and control all-malt worts (Standard FAN) decreased, and at 192 hours the concentrations were almost equal, suggesting that increasing the concentration of preferred amino acids, i.e. those typically absorbed quicker than valine into the cell, did not have as large of an effect on valine uptake rate as increasing the amount of non-preferred amino acids, i.e. those typically absorbed slower than valine into the cell. Again the diacetyl reduction rate at the end of fermentation was larger in the all-malt wort fermentations, which can be explained by the fact that more biomass was available for reduction.

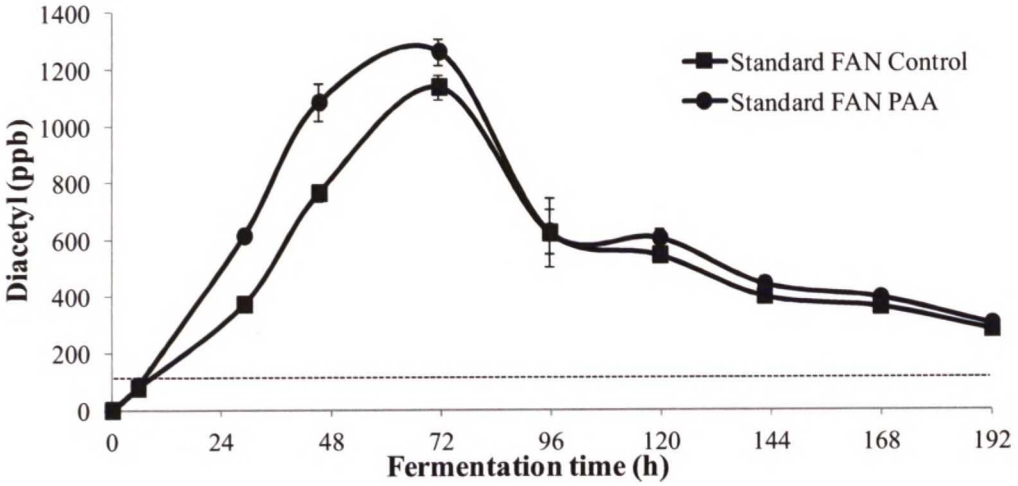

Figure 36 – The diacetyl concentration (ppb or µg/l) of the all-malt worts (Standard FAN) used for the second experimental fermentations of the third experiment as a function of fermentation time (hours). The dotted line at 100 ppb depicts the flavour threshold. Error bars where visible represent standard deviation of sample.

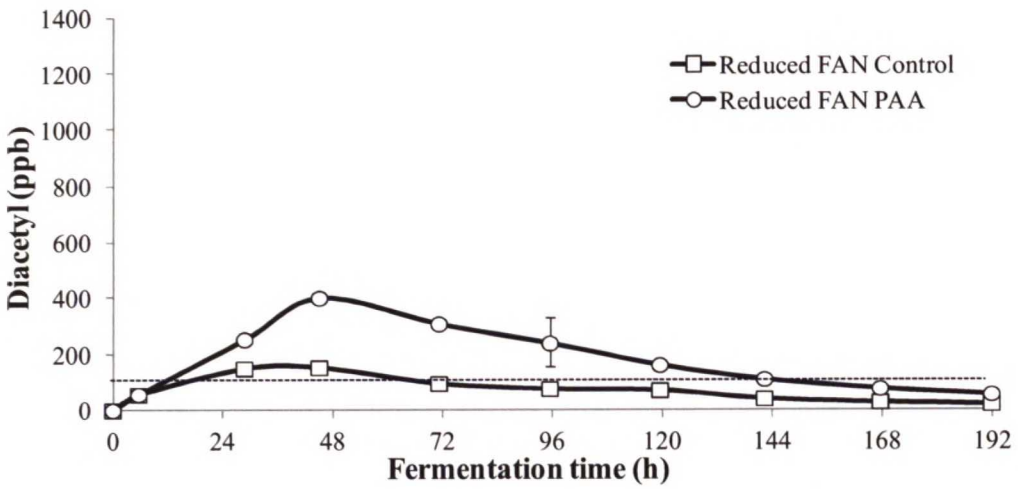

Figure 37 – The diacetyl concentration (ppb or µg/l) of the semi-synthetic worts (Reduced FAN) used for the second experimental fermentations of the third experiment as a function of fermentation time (hours). The dotted line at 100 ppb depicts the flavour threshold. Error bars where visible represent standard deviation of sample.

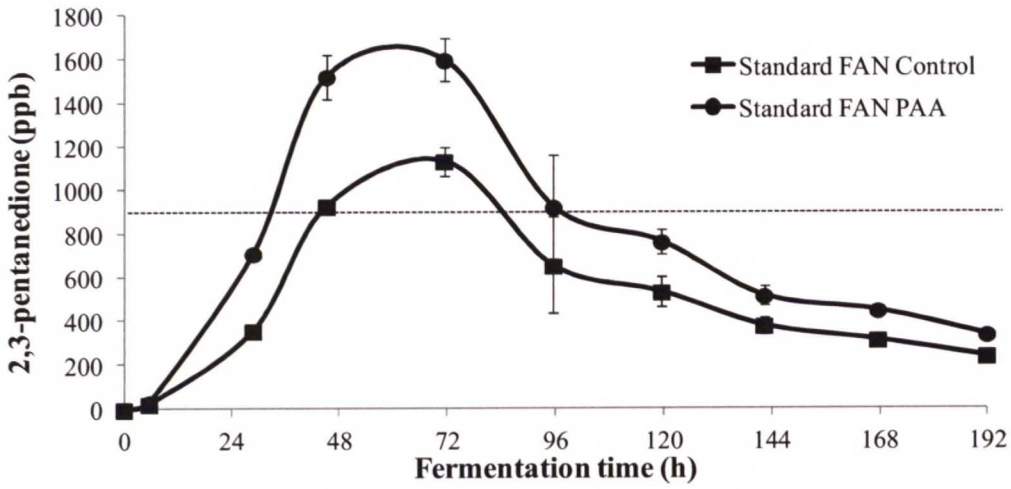

Figure 38 – The 2,3-pentanedione concentration (ppb or µg/l) of the all-malt worts (Standard FAN) used for the second experimental fermentations of the third experiment as a function of fermentation time (hours). The dotted line at 900 ppb depicts the flavour threshold. Error bars where visible represent standard deviation of sample.

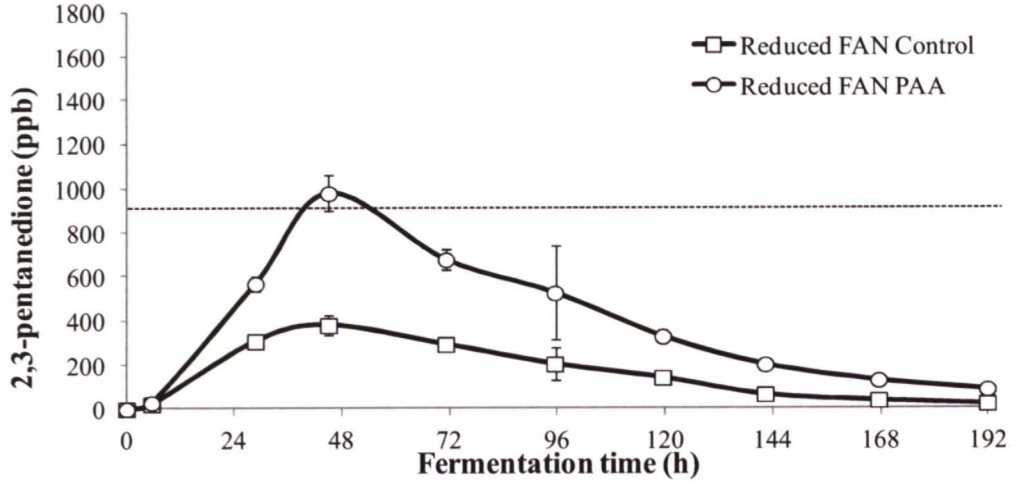

Figure 39 – The 2,3-pentanedione concentration (ppb or µg/l) of the semi-synthetic worts (Reduced FAN) used for the second experimental fermentations of the third experiment as a function of fermentation time (hours). The dotted line at 900 ppb depicts the flavour threshold. Error bars where visible represent standard deviation of sample.

4.3.3 Amino acid uptake

The concentrations of valine, isoleucine and leucine in the fermenting all-malt wort (Standard FAN) of the first experimental fermentations as a function of fermentation time are presented in Figure 40, Figure 41, and Figure 42 respectively. As can be seen from the plots, the valine uptake rate of the yeast was decreased during whole fermentation for the wort supplemented with branched-chain amino acids, while the uptake rate in the wort supplemented with non-preferred amino acids initially increased, but later in the fermentation decreased, compared to the control wort. At the end of fermentation, the control wort reached the lowest valine concentration, while the valine concentrations in the BCAA- and NPAA-supplemented worts were similar. The uptake rate of isoleucine by the yeast in both the BCAA- and NPAA-supplemented worts (isoleucine included in both amino acid groups) was increased compared to that of the control wort, while the uptake rate of leucine increased in the BCAA-supplemented wort and decreased in the NPAA-supplemented wort (leucine included only in the BCAA group), compared to the control wort.

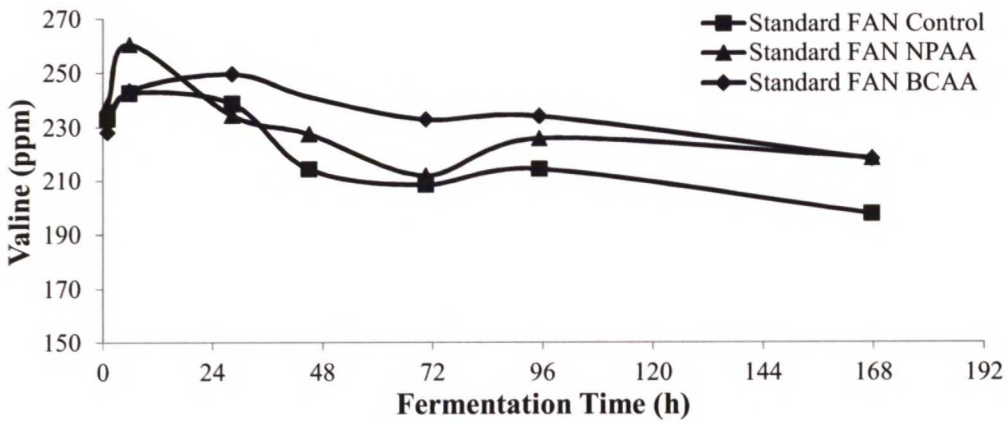

Figure 40 – The valine concentration (ppm or mg/l) of the all-malt worts (Standard FAN) used for the first experimental fermentations of the third experiment as a function of fermentation time (hours). The relative standard deviation of the amino acid concentrations was below 2.2% for all measurements.

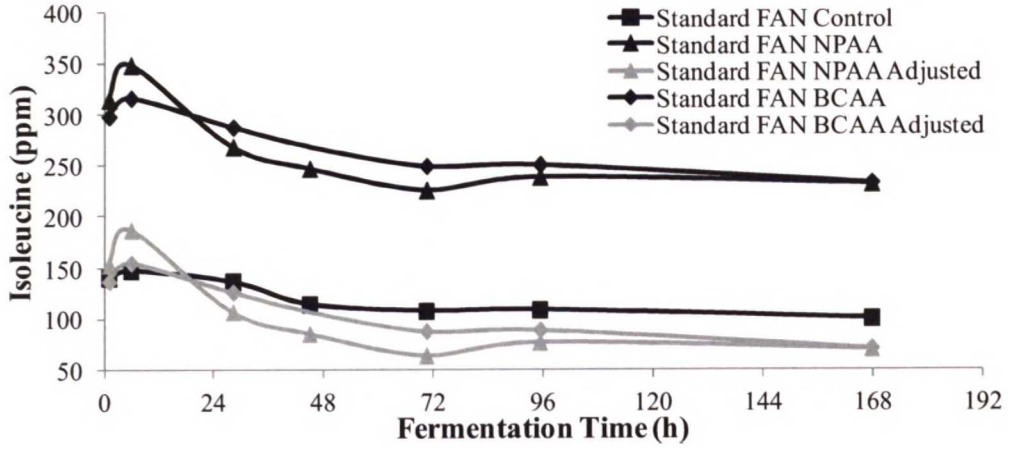

Figure 41 – The isoleucine concentration (ppm or mg/l) of the all-malt worts (Standard FAN) used for the first experimental fermentations of the third experiment as a function of fermentation time (hours). The adjusted plots contain the values of their respective plot with 161.2 ppm subtracted, to facilitate comparison between the slopes. The relative standard deviation of the amino acid concentrations was below 2.2% for all measurements.

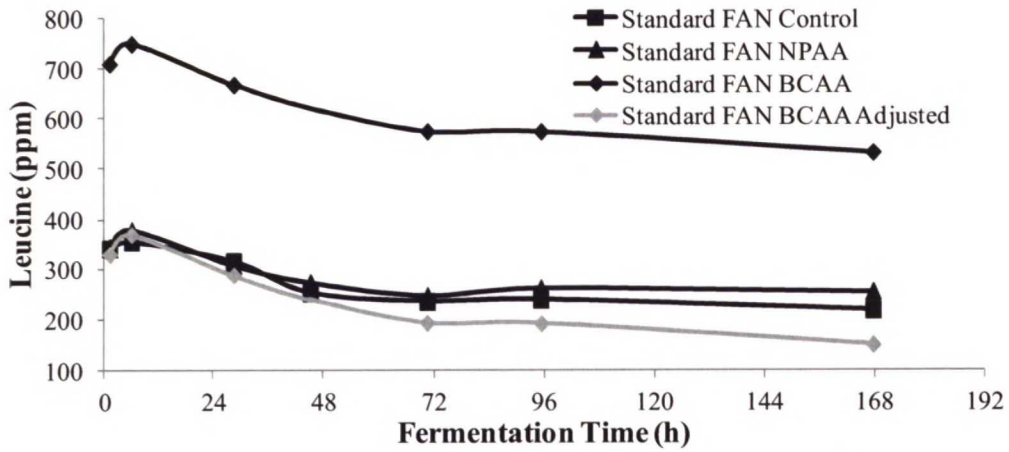

Figure 42 – The leucine concentration (ppm or mg/l) of the all-malt worts (Standard FAN) used for the first experimental fermentations of the third experiment as a function of fermentation time (hours). The adjusted plot contains the values of the 'Standard FAN BCAA' plot with 380.3 ppm subtracted, to facilitate comparison between the slopes. The relative standard deviation of the amino acid concentrations was below 2.2% for all measurements.

A combined plot of the change in valine concentration (i.e. valine uptake rate; ppm/h) and the change in diacetyl concentration (i.e. the diacetyl production rate; ppb/h) of the fermenting all-malt worts (Standard FAN) of the first experimental fermentations as a function of fermentation time is presented in Figure 43. Similar to the results from the first experiment, it can be seen from the plot that when valine uptake was high in the beginning of the fermentation, diacetyl production rate was concurrently low. The valine uptake rate of the yeast was higher (i.e. the change in valine concentration is more negative) and diacetyl production rate lower in the NPAA-supplemented wort, compared to the control wort during the first 20 hours of fermentation, while a lower valine uptake and higher diacetyl production was observed in the BCAA-supplemented wort. Between 24 and 48 hours, the diacetyl production rate of the NPAA-supplemented wort was higher and the valine uptake rate lower than that of the control wort, while the BCAA-supplemented wort displayed similar trends up to 40 hours, after which there was a lower diacetyl production rate despite the lower valine uptake rate. The results suggest that valine uptake rate negatively correlates with the amount of diacetyl formed during the growth phase of fermentation with certain exceptions.

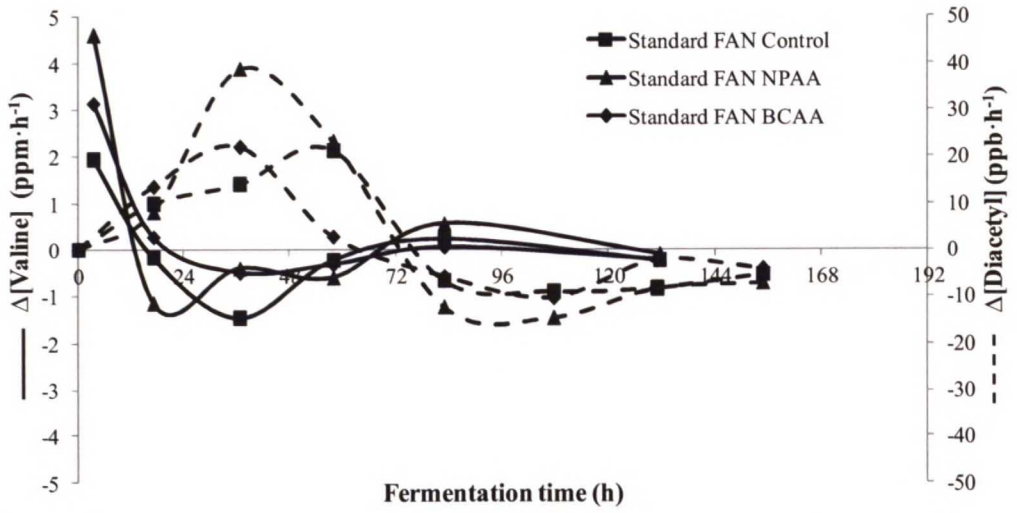

Figure 43 – The average linear change in wort valine concentration (ppm/h) and diacetyl concentration (ppb/h) of the all-malt worts (Standard FAN) used for the first experimental fermentations of the third experiment as a function of fermentation time (hours).

The concentrations of valine, isoleucine and leucine in the fermenting all-malt wort (Standard FAN) of the second experimental fermentations as a function of fermentation time are presented in Figure 44, Figure 45, and Figure 46 respectively. As can be seen from the plots, the valine and isoleucine uptake rate of the yeast was decreased during the first 72 hours of fermentation for the wort supplemented with the preferred amino acids compared to the control wort, while the uptake rate of leucine remained similar. Towards the end of fermentation on the other hand, the uptake rate of valine and isoleucine was slightly higher in the PAA-supplemented wort compared to the control wort. The difference between the initial and final valine and isoleucine concentration in the wort was greater for the control wort compared to the PAA-supplemented wort.

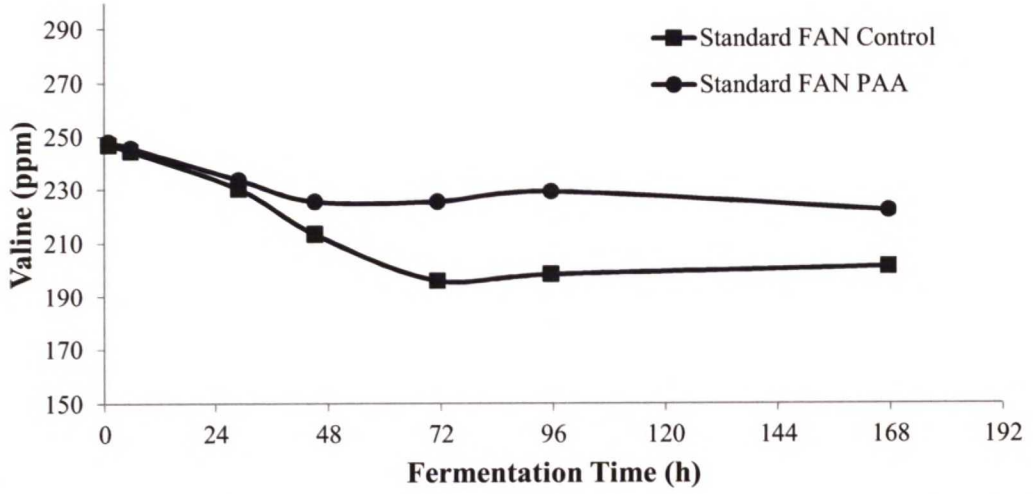

Figure 44 – The valine concentration (ppm or mg/l) of the all-malt worts (Standard FAN) used for the second experimental fermentations of the third experiment as a function of fermentation time (hours). The relative standard deviation of the amino acid concentrations was below 2.2% for all measurements.

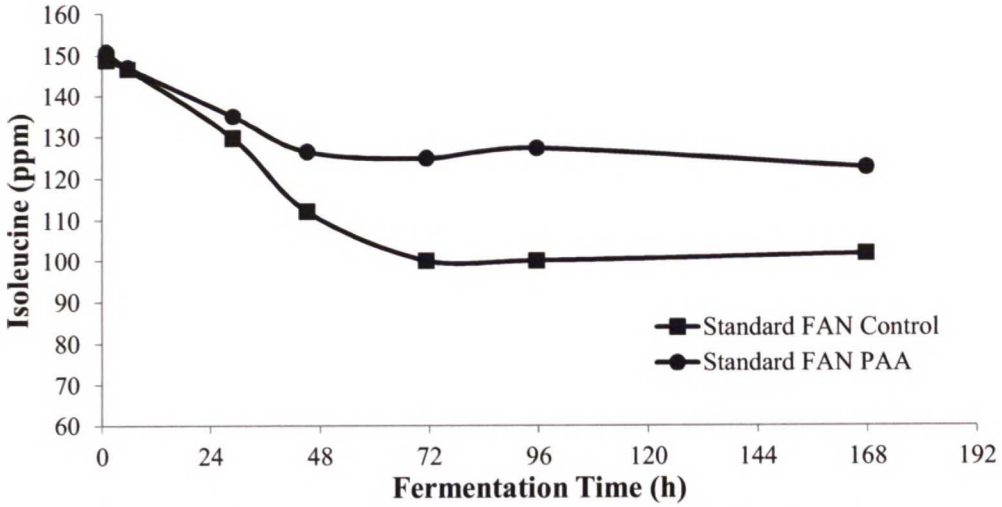

Figure 45 – The isoleucine concentration (ppm or mg/l) of the all-malt worts (Standard FAN) used for the second experimental fermentations of the third experiment as a function of fermentation time (hours). The relative standard deviation of the amino acid concentrations was below 2.2% for all measurements.

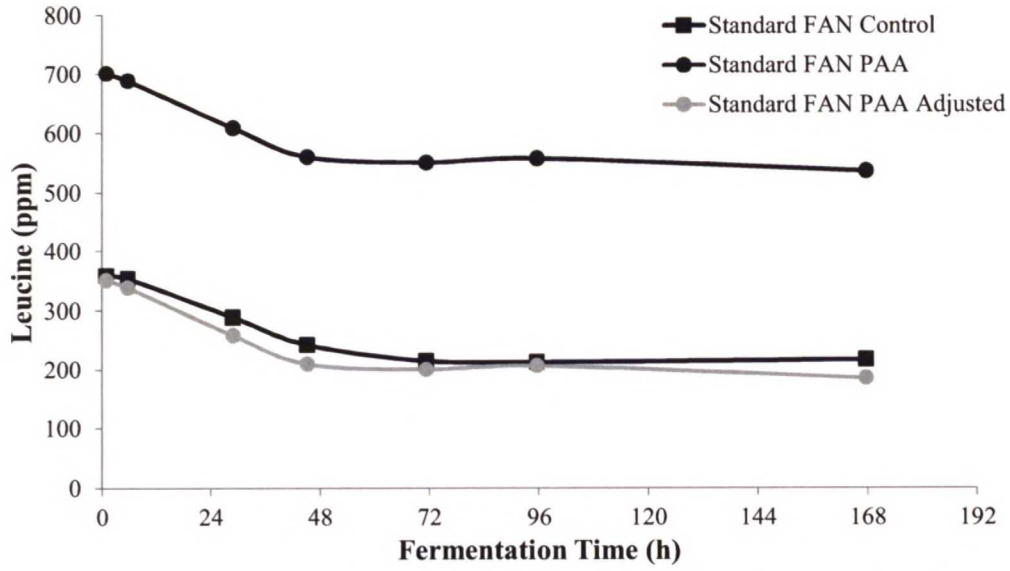

Figure 46 – The leucine concentration (ppm or mg/l) of the all-malt worts (Standard FAN) used for the second experimental fermentations of the third experiment as a function of fermentation time (hours). The adjusted plot contains the values of the 'Standard FAN PAA' plot with 350 ppm subtracted, to facilitate comparison between the slopes. The relative standard deviation of the amino acid concentrations was below 2.2% for all measurements.

A combined plot of the change in valine concentration (i.e. valine uptake rate; ppm/h) and the change in diacetyl concentration (i.e. the diacetyl production rate; ppb/h) of the fermenting all-malt worts (Standard FAN) of the second experimental fermentations as a function of fermentation time is presented in Figure 47. As can be seen from the plot, the valine uptake rate of the yeast is lower (i.e. the change in valine concentration is less negative) in the PAA-supplemented wort compared to the control wort for the majority of the fermentation. At the beginning of fermentation, the diacetyl production rate of the yeast is higher in the PAA-supplemented wort compared to the control wort, again suggesting that valine uptake rate negatively correlates with the amount of diacetyl produced during the growth phase of fermentation, but towards the end of fermentation, the diacetyl production rate becomes similar, and at times lower, to that of the yeast in the control wort, despite the lower valine uptake rate.

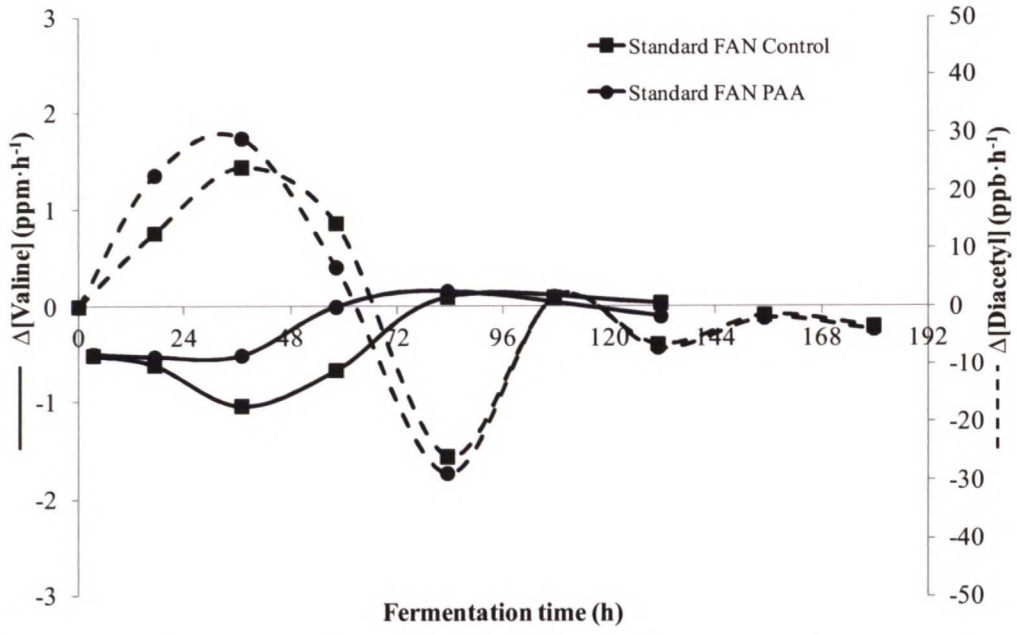

Figure 47 – The average linear change in wort valine concentration (ppm/h) and diacetyl concentration (ppb/h) of the all-malt worts (Standard FAN) used for the second experimental fermentations of the third experiment as a function of fermentation time (hours).

4.3.4 Aroma compounds

The concentrations of aroma compounds in the beers (samples taken after 168 hours of fermentation) produced during the first experimental fermentations of the third experiment, as well as their typical flavour thresholds (Meilgaard, 1982; Siebert, 1988), are presented in Table 19. The concentration of 2-methylpropanol, formed from valine, is similar in the three all-malt worts, but slightly higher in the semi-synthetic control wort compared to the NPAA- and BCAA-supplemented semi-synthetic worts. The concentrations of 3-methylbutanol, formed from leucine, and 3-methylbutylacetate (the acetate ester of 3-methylbutanol) in both BCAA-supplemented worts were higher than the control and NPAA-supplemented worts, suggesting that leucine uptake rate of the yeast is positively correlated with the amount of 3-methylbutanol produced during fermentation, which further correlates with the amount of 3-methylbutylacetate produced. Similar results were obtained with the concentrations of 2-methylbutanol, formed from isoleucine, where higher concentrations of 2-methylbutanol were observed in the beer fermented from the BCAA- and NPAA-supplemented worts compared to control wort, with the exception of the BCAA-supplemented all-malt wort, which had a lower concentration of 2-methylbutanol compared to the control wort. This could possibly be explained by the similar isoleucine uptake rates during the first 24 hours of fermentation between the BCAA-supplemented and control all-malt worts. Of the examined alcohols, only 2-methylbutanol was found in concentrations above its flavour threshold, and that only in the NPAA-supplemented wort. The concentrations of several of the examined esters were above or around their flavour thresholds, but supplementation of the amino acid groups had no effect or even decreased the concentrations in most cases, with 3-methylbutylacetate in the BCAA-supplemented worts being the only exception.

Table 19 – The concentrations of aroma compounds in the beer from the first experimental fermentations of the third experiment (ppm or mg/l) and their typical flavour thresholds (Meilgaard, 1982; Siebert, 1988). Values are means from two independent fermentations. Error represents standard deviation of mean.

Compound (ppm)	Standard FAN			Reduced FAN			Flavour Threshold (ppm)
	Control	NPAA	BCAA	Control	NPAA	BCAA	
Acetaldehyde	19.4 (± 2.6)	18.5 (± 2.0)	18.0 (± 1.9)	10.5 (± 1.6)	10.6 (± 1.0)	10.5 (± 1.0)	10
1-Propanol	10.2 (± 0.3)	8.54 (± 0.13)	8.32 (± 0.12)	16.3 (± 0.6)	11.2 (± 0.3)	10.9 (± 0.5)	800
2-Methylpropanol	19.8 (± 0.3)	18.5 (± 0.3)	16.2 (± 0.3)	34.1 (± 1.7)	23.6 (± 0.3)	22.0 (± 0.2)	200
3-Methylbutanol	28.8 (± 1.2)	25.6 (± 0.7)	39.8 (± 0.7)	29.8 (± 0.9)	26.0 (± 0.7)	55.3 (± 0.7)	70
2-Methylbutanol	49.0 (± 1.7)	59.2 (± 1.7)	48.7 (± 1.3)	48.6 (± 1.8)	65.1 (± 1.8)	62.1 (± 0.9)	65
2-Phenylethylalcohol	1.62 (± 0.21)	2.48 (± 0.82)	2.04 (± 0.74)	1.85 (± 0.07)	1.94 (± 0.09)	1.88 (± 0.18)	100 – 125
Ethyl acetate	24.2 (± 0.9)	24.6 (± 1.8)	23.4 (± 2.1)	23.8 (± 1.8)	23.3 (± 1.1)	23.7 (± 1.8)	30 – 33
Ethyl caproate	0.29 (± 0.03)	0.30 (± 0.03)	0.27 (± 0.03)	0.32 (± 0.02)	0.29 (± 0.02)	0.31 (± 0.02)	0.21 – 0.23
Ethyl caprylate	0.83 (± 0.18)	0.93 (± 0.11)	0.86 (± 0.14)	0.83 (± 0.14)	0.78 (± 0.14)	0.82 (± 0.12)	0.9
Ethyldecanoate	2.74 (± 2.05)	1.90 (± 0.46)	1.85 (± 0.29)	1.42 (± 0.19)	1.05 (± 0.13)	1.16 (± 0.20)	1.5
3-Methylbutylacetate	2.26 (± 0.10)	1.93 (± 0.11)	3.09 (± 0.36)	2.00 (± 0.12)	1.53 (± 0.06)	3.69 (± 0.24)	1.2 – 1.6
2-Phenylethylacetate	0.48 (± 0.41)	0.20 (± 0.03)	0.21 (± 0.03)	0.19 (± 0.01)	0.18 (± 0.01)	0.18 (± 0.01)	3.8

The concentrations of aroma compounds in the beers (samples taken after 168 hours of fermentation) produced during the second experimental fermentations of the third experiment, as well as their typical flavour thresholds (Meilgaard, 1982; Siebert, 1988), are presented in Table 20. The concentrations of 2-methylpropanol (formed from valine) and 2-methylbutanol (formed from isoleucine) are lower in the PAA-supplemented wort compared to the control wort, while the concentrations of 3-

methylbutanol (formed from leucine) and 3-methylbutylacetate (the acetate ester of 3-methylbutanol) are higher in the PAA-supplemented wort compared to the control wort. These results again suggest a positive correlation between the uptake rate of branched-chain amino acids and the formation of their respective higher alcohol formed via transamination. The concentrations of the examined alcohols in the beers from second experimental fermentations of the third experiment were all below their flavour thresholds, while the concentrations of several of the examined esters were above or around their flavour thresholds. Supplementation of the PAA had little effect on the concentrations of esters in the all-malt worts, with the exception of 3-methylbutylacetate, while slight increases in the examined esters were observed for the supplemented semi-synthetic worts.

Table 20 – The concentrations of aroma compounds in the beer from the second experimental fermentations of the third experiment (ppm or mg/l) and their typical flavour thresholds (Meilgaard, 1982; Siebert, 1988). Values are means from two independent fermentations. Error represents standard deviation of mean.

Compound (ppm)	Standard FAN		Reduced FAN		Flavour Threshold (ppm)
	Control	PAA	Control	PAA	
Acetaldehyde	18.0 (± 0.8)	19.2 (± 1.2)	9.8 (± 0.38)	14.4 (± 0.9)	10
1-Propanol	11.0 (± 0.7)	11.0 (± 0.7)	17.3 (± 1.0)	15.6 (± 1.2)	800
2-Methylpropanol	18.8 (± 0.2)	15.6 (± 0.4)	37.3 (± 0.8)	17.4 (± 0.6)	200
3-Methylbutanol	30.8 (± 0.8)	39.8 (± 1.3)	31.3 (± 0.6)	39.0 (± 1.9)	70
2-Methylbutanol	49.6 (± 1.3)	42.7 (± 0.9)	48.9 (± 1.0)	38.4 (± 1.0)	65
2-Phenylethylalcohol	2.39 (± 1.34)	2.58 (± 1.16)	2.26 (± 0.38)	2.21 (± 0.14)	100 – 125
Ethyl acetate	24.2 (± 1.2)	22.8 (± 3.1)	22.4 (± 1.0)	23.2 (± 1.1)	30 – 33
Ethyl caproate	0.30 (± 0.03)	0.27 (± 0.06)	0.33 (± 0.02)	0.35 (± 0.02)	0.21 – 0.23
Ethyl caprylate	0.94 (± 0.20)	0.91 (± 0.23)	0.92 (± 0.14)	1.05 (± 0.19)	0.9
Ethyldecanoate	1.71 (± 0.72)	1.44 (± 0.22)	1.14 (± 0.20)	1.27 (± 0.36)	1.5
3-Methylbutylacetate	2.37 (± 0.06)	2.93 (± 0.56)	1.93 (± 0.04)	2.46 (± 0.14)	1.2 – 1.6
2-Phenylethylacetate	0.30 (± 0.25)	0.23 (± 0.04)	0.20 (± 0.03)	0.22 (± 0.05)	3.8

4.4 Effects of supplementing valine to all-malt wort and pre-conditioning yeast in a valine/glucose solution on beer quality and the production of diacetyl during fermentation

4.4.1 Fermentation performance

The ethanol content (% v/v) and the estimated real extracts (weight %) of the worts used for the experimental fermentations of the fourth experiment as a function of fermentation time are presented in Figure 48. As can be seen from the plot, the worts fermented with pre-conditioned yeast attenuated slightly faster than the worts fermented with the control yeasts, as 80% apparent attenuation was reached about 20 hours earlier in them. Valine supplementation appears to have had no effect on either fermentation rate or final attenuation level of the pre-conditioned yeast, but valine supplementation to the control yeast decreased its fermentation performance slightly, an effect not seen in any previous fermentations. After fermentation had finished, all beers contained an ethanol content of around 6.7% (v/v). The yeast dry mass and fresh mass content of the worts (g/l) as a function of fermentation time is presented in Figure 49, and as can be seen from the plot, valine supplementation appears to have had no effect on the amount of yeast biomass produced during fermentation for the pre-conditioned yeasts either, while there was a slight decrease in amount of biomass produced for the valine-supplemented control yeast. The maximum biomass observed during fermentation was higher for the pre-conditioned yeasts compared to the control yeasts. The pH of the valine-supplemented worts remained similar for all treatments throughout the fermentation as well (see Figure 3 in Appendix 3), suggesting that fermentation performance was not affected by the supplementation of various amounts of valine to the wort.

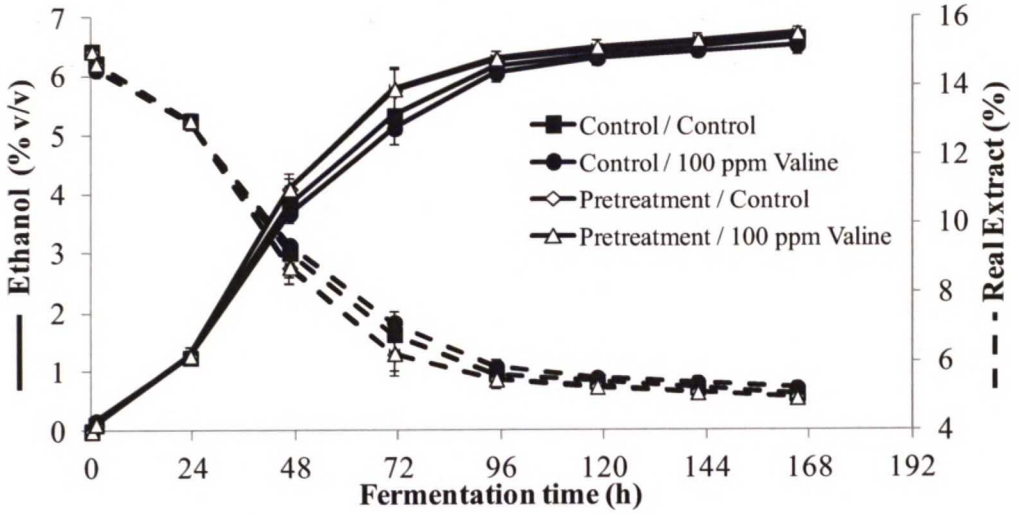

Figure 48 – The ethanol content (% v/v; solid line) and real extract (weight %; dashed line) of the worts used for the experimental fermentations of the fourth experiment as a function of fermentation time (hours). Values are means from two independent fermentations. Error bars where visible represent standard deviation of mean.

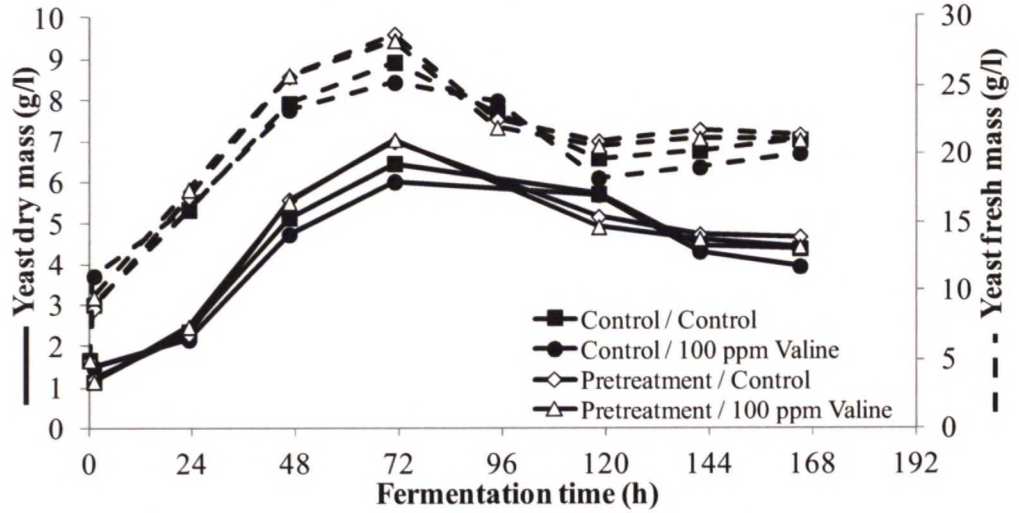

Figure 49 – The yeast dry mass content (g/l; solid line) and yeast fresh mass content (g/l; dashed line) of the worts used for the experimental fermentations of the fourth experiment as a function of fermentation time (hours). Values are means from two independent fermentations. Error bars where visible represent standard deviation of mean.

4.4.2 VDK production

The concentrations of diacetyl and 2,3-pentanedione in the fermenting worts used for the experimental fermentations of the fourth experiment and the matured beer at different time points are presented in Figure 50 and Figure 51 respectively. As can be seen, the diacetyl concentrations at 71 hours were higher in the worts not supplemented with valine compared to those supplemented with valine, and also higher in the worts fermented with the pre-conditioned yeast compared to the control yeast. At the end of primary fermentation (165 hours), the difference in diacetyl concentrations between the worts decreased (maximum difference 71 ppb), and in the matured, but undiluted, beer the difference decreased even further (maximum difference 27 ppb).

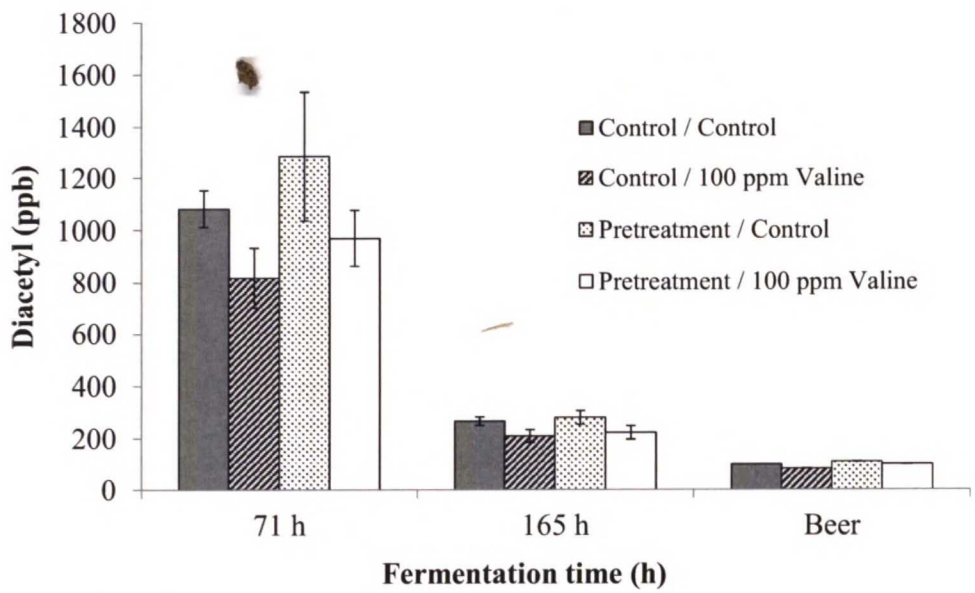

Figure 50 – The diacetyl concentration (ppb or µg/l) of the all-malt worts used for the experimental fermentations and the matured beer of the fourth experiment at 71 and 165 hours. Values are means from two independent fermentations. Error bars where visible represent standard deviation of mean.

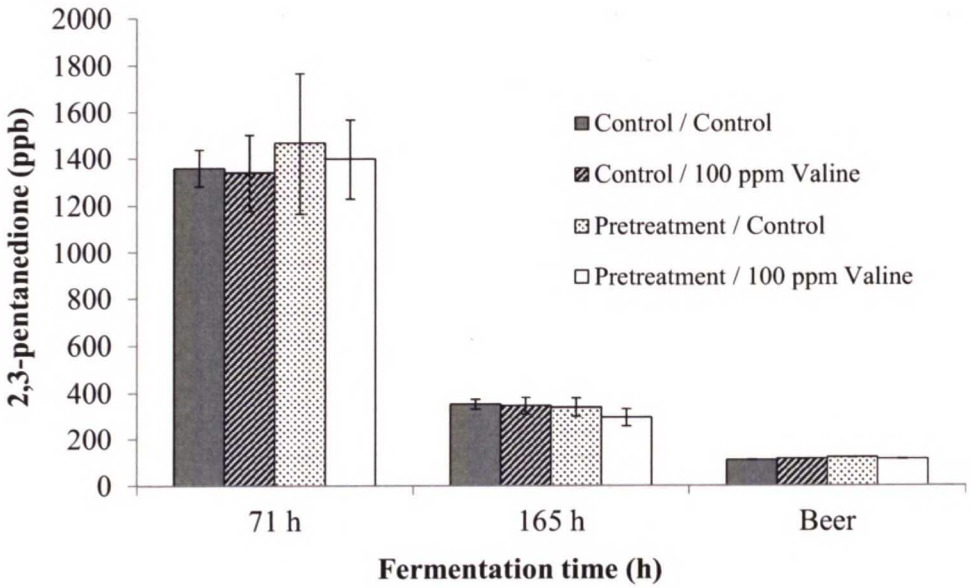

Figure 51 – The 2,3-pentanedione concentration (ppb or µg/l) of the all-malt worts used for the experimental fermentations and the matured beer of the fourth experiment at 71 and 165 hours. Values are means from two independent fermentations. Error bars where visible represent standard deviation of mean.

4.4.3 Foam stability

The foam stabilities of the beers produced from the fermentations of the fourth experiment are presented in Figure 52. The foam stabilities of the beers were very similar, and valine supplementation to the pre-conditioned yeast just slightly increased overall foam stability, while valine supplementation to the control yeast just slightly decreased overall foam stability.

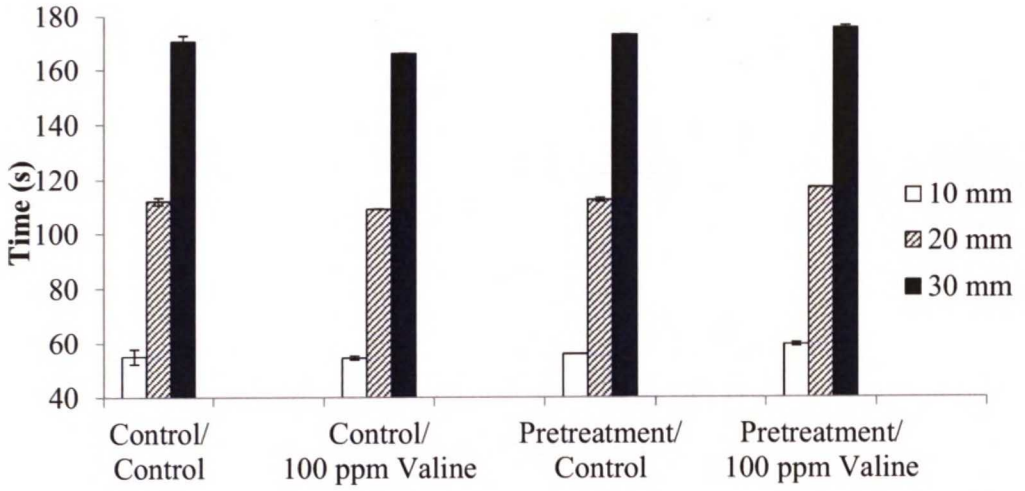

Figure 52 – Foam stabilities of the beers produced from the fermentations of the fourth experiment. Bars represent the time taken (seconds) for the foam to collapse 10, 20 and 30 mm.

5 Discussion

The purpose of this study was to investigate whether the supplementation of valine to brewer's wort or the modification of wort amino acid profile could influence the amount of vicinal diketones produced during fermentation. By supplementing valine to brewer's wort, it was possible to decrease both the maximum diacetyl concentration observed during fermentation and the diacetyl concentration at the end of fermentation, suggesting that beer maturation times could be shortened, which in turn could benefit breweries economically. The composition of the amino acid spectrum of the wort also had a significant impact on diacetyl and 2,3-pentanedione production during fermentation, suggesting that diacetyl production could be reduced by modifying the wort amino acid spectrum through raw material choices, adjuncts, malting conditions, mashing conditions, or even side-stream refinement. The results from all four experiments showed that fermentation performance and yeast growth are not affected by the studied levels of amino acid supplementations, implying that supplementation of valine or other amino acids will not affect the primary fermentation time nor the attenuation level achieved.

Previous studies on the subject have shown that higher wort valine concentrations and greater valine uptake result in decreased diacetyl production during fermentation (Cyr *et al.*, 2007; Nakatani *et al.*, 1984; Petersen *et al.*, 2004). However, no previous trials seem to have been performed to determine the effects of alteration of background wort amino acid profile on the production of diacetyl during fermentation. The results from the first, second and fourth experiment all agree in that an increased initial wort valine concentration, through direct supplementation, resulted in a lower maximum diacetyl concentration observed during fermentation and lower diacetyl concentrations at the end of fermentation (see Figures 10, 14 and 15). The changes of wort valine concentration during fermentations of the second experiment (see Figure 18) show that supplementation of valine resulted in an increased uptake rate of valine into the yeast cells, suggesting that the decreased diacetyl concentrations during fermentation are a result of less pyruvate being converted into α-acetolactate because of the inhibition of AHAS by valine (Magee

and de Robichon-Szulmajster, 1968). These results agree with those presented by Nakatani *et al.* (1984), where increased amounts of valine supplementation resulted in increased valine uptake and decreased maximum valine concentrations observed during fermentation.

According to the results observed by Didion *et al.* (1996), valine does not have any significant inducing effect on the expression of *BAP2* in *S. cerevisiae*, while de Boer *et al.* (1998) observed that a number of amino acids, not only branched-chained amino acids, induced the expression of *BAP3* in *S. cerevisiae*. This suggests that increased expression of specific branched-chain amino acid permease-encoding genes may only be a minor cause of the increased valine uptake caused by valine supplementation. Rather, since the uptake rate of leucine and isoleucine slightly decreased in the beginning of the fermentation, the increased valine uptake rate can most likely be explained by increased interactions between valine and the amino acid permeases caused by the increased ratio of valine to other branched-chain amino acids following valine supplementation. 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 (Nielsen *et al.*, 2001). By studying the effect that valine or other branched-chain amino acids have on the expression of amino acid permeases such as *BAP2*, *BAP3*, *TAT1* and *GAP1*, e.g. by constructing reporter strains expressing green fluorescent protein (GFP) linked to the permease promoters, real-time quantitative PCR, or utilizing transcript analysis with aid of affinity capture (TRAC; Rautio *et al.*, 2006), better knowledge could be obtained on the timing of amino acid absorption and how the amino acid profile affects the uptake of individual amino acids. Amino acid uptake could also be measured directly through the use of radiolabelled amino acids (Regenberg *et al.*, 1999).

The results from the second experiment suggest that lowering the free amino nitrogen content of wort could decrease the amount of diacetyl produced during fermentation despite a reduced valine concentration, but fermentation performance

was simultaneously degraded as a result of decreased yeast growth (see Figures 12-15). Pugh *et al.* (1997) also observed decreased diacetyl concentrations with wort FAN content decreasing from 216 ppm to 144 ppm, after which diacetyl concentrations increased again as FAN content was decreased to 122 ppm. Nakatani *et al.* (1984) on the other hand report a negative correlation between the initial wort FAN content and the maximum VDK concentration observed during fermentation. Lei *et al.* (2013) 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 increased in total valine concentration. The valine concentrations in the semi-synthetic worts were not analysed, but the decreased diacetyl production of the semi-synthetic worts was most likely caused by an increased valine uptake rate resulting from the lower concentrations of other amino acids absorbed into the yeast with higher affinity than valine. Hence, it becomes evident that it is not the valine concentration *per se* that is of central importance regarding the production rate of diacetyl during fermentation, but rather the uptake rate of valine. Since the pH of the semi-synthetic worts was lower during fermentation than that of the all-malt worts, most likely due to loss of buffer capacity through dilution, the lower diacetyl concentrations produced with wort containing a reduced FAN content may also be influenced in this case by an increased reaction rate of the rate-limiting spontaneous decarboxylation of α-acetolactate into diacetyl, as the reduction of diacetyl to acetoin and 2,3-butanediol is rapid (Boulton and Box, 2003; Garcia *et al.*, 1994; Kobayashi *et al.*, 2005a; Rondags *et al.*, 1996).

Examining the results from the third experiment it becomes evident that the concentrations of other amino acids in the wort have an impact on the valine uptake and diacetyl production by yeast (see Figures 32, 33, 36 and 37). Supplementing the preferred amino acids, i.e. those that had been absorbed in larger amounts than valine during the first 25 hours of fermentation, to all-malt wort resulted in an increased difference in diacetyl concentration compared to the control wort in the beginning of fermentation (until 72 hours), after which it decreases considerably towards a negligible difference at the end of fermentation (see Figure 36). Supplementing the

non-preferred amino acids, i.e. those that had been absorbed in lesser amounts than valine during the first 25 hours of fermentation, to all-malt wort on the other hand, resulted in negligible difference in wort diacetyl concentration during the first 24 hours of fermentation compared to the control wort, followed by an increased difference mid-fermentation (24 to 120 hours), after which the difference starts decreasing again towards the end of fermentation. The NPAA-supplemented semi-synthetic wort also had a much later and larger diacetyl peak compared to the of the semi-synthetic control wort (Figure 33).

These results suggest that increased wort concentration of amino acids that are quickly absorbed have less effect on the diacetyl concentration at the end of fermentation and instead result in an initial increase in diacetyl production as well as an early diacetyl peak, since they affect valine uptake mostly at the beginning of the fermentation. Increased wort concentrations of amino acids that are slowly absorbed by the yeast, and hence remain longer in the wort and compete with valine for transporters, have a larger effect on the diacetyl concentration at the end of fermentation and result in increased diacetyl production towards the middle of fermentation and thus a later diacetyl peak. Looking at the change in wort valine concentration of the NPAA- and PAA-supplemented worts compared to their control worts (see Figures 43 and 47), it becomes apparent that there was a larger decrease in wort valine from the initial valine concentration in the NPAA-supplemented wort compared to the control wort during the first 24 hours of fermentation, despite a larger amount of amino acids present in the wort competing for permease interactions. Reasons for this may be an overall increase in expression of genes encoding amino acid permeases, e.g. through sensing by Ssy1p (Didion *et al.*, 1998), yet it is unlikely that the effects would have that noticeable of an effect in the relatively amino acid-rich wort. It was noted that the amino acids in the preferred amino acid group were almost all polar by nature, with the exception of leucine and phenylalanine, suggesting that transport of polar amino acids across the cell membrane might be more efficient. No studies were however found in literature to confirm this and it may be a mere coincidence.

The results from the third experiment also suggest that increased uptake rates of other branched-chain amino acids than valine, i.e. leucine and isoleucine, can also potentially decrease the production rate of diacetyl during fermentation (see Figures 32-33). Since the maximum diacetyl concentration and diacetyl concentration at the end of fermentation was lower in the BCAA-supplemented all-malt wort compared to the control wort, and the valine uptake was significantly decreased in the BCAA-supplemented wort compared to the control wort (see Figure 40), it is evident that valine is not the only amino acid responsible for reduced diacetyl production. Studies have shown varying data on the inhibiting effect of other branched-chain amino acids on AHAS, as both Barton and Slaughter (1992) and Magee and de Robichon-Szulmajster (1968) observed that leucine also had an inhibiting effect on the AHAS enzyme's ability to produce α-acetolactate from pyruvate, though not as strong as the inhibiting effect of valine, while no inhibiting effect of isoleucine on the AHAS enzyme's ability to produce α-acetolactate from pyruvate was found. Pang and Duggleby (2001) observed the opposite, that isoleucine had a slight inhibiting effect and leucine had no inhibiting effect on the AHAS enzyme's ability to produce α-acetolactate from pyruvate. To obtain better knowledge on the contribution of individual amino acids on the production of diacetyl or its precursor α-acetolactate, the effect of individual amino acids on both AHAS activity and *ILV2* expression could be measured, e.g. with an enzyme assay (Byrne and Meacock, 2001), construction of reporter strains expressing GFP linked to the *ILV2* promoter, real-time quantitative PCR, or utilizing TRAC. The uptake rate of both isoleucine and leucine were increased in the BCAA-supplemented wort compared to the control wort (see Figures 41-42), suggesting that the lower diacetyl production observed in the BCAA-supplemented wort could result from AHAS inhibition by leucine. This might also explain the similar diacetyl production rates towards the latter half of fermentation observed in the PAA-supplemented wort, containing increased concentrations of leucine, and its control wort. The initial diacetyl production rate of the BCAA-supplemented wort was however higher than the control wort, which presumably is a result of a combination of lower inhibiting effect on AHAS of leucine than valine and the decreased total uptake rate of branched-chain amino acids during the first approximately 12 hours of fermentation, most likely caused by the

increased competition for permease interactions. The total uptake rate of branched-chain amino acids increased though towards the middle of fermentation in the BCAA-supplemented wort, perhaps from increased expression of genes encoding amino acid permeases (e.g. *BAP2* and *BAP3*) as a result of increased amino acid concentrations (Didion *et al.*, 1996).

Nakatani *et al.* (1984) observed that supplementation of increasing amounts of isoleucine to wort increased diacetyl formation in the beginning of fermentation, but resulted in earlier diacetyl peaks and lower diacetyl concentrations at the end of fermentation, caused by the postponing of valine uptake in the worts supplemented with high amounts of isoleucine and depletion of valine in the control wort and the worts supplemented with lower amounts of isoleucine. Hence, isoleucine does not have any direct effect on diacetyl production, but rather decreases the uptake rate of valine and leucine, through shared amino acid permeases. Similar results were observed with the Reduced FAN fermentations of the third experiment, where fermentation of the BCAA-supplemented semi-synthetic wort produced a higher maximum diacetyl concentration, yet a lower diacetyl concentration at the end of fermentation compared to the control wort (see Figure 33), as a result of a 'double peak' diacetyl profile generated by the control wort. Thus, when striving for the lowest possible diacetyl concentration at the end of fermentation and the wort contains such a low initial FAN content that valine is either depleted from the wort or valine uptake rate is significantly impaired during fermentation, postponed valine uptake might even be desired.

Supplementation of the amino acid groups containing isoleucine, i.e. NPAA and BCAA, resulted in lowered 2,3-pentanedione concentrations compared to the control worts throughout the fermentation period for all examined fermentations. Because of the approximately 10-fold flavour threshold of 2,3-pentanedione compared to diacetyl though, the lowered 2,3-pentanedione concentrations will not have a large impact on beer quality, since the 2,3-pentanedione concentrations in the control and PAA-supplemented worts were only above the flavour thresholds in the early half of fermentations. Hence the advantages gained from decreased 2,3-pentanedione

concentrations following increased isoleucine uptake, do not outweigh the disadvantages gained from potential increased diacetyl concentrations resulting from decreased valine and leucine uptake rate. Results from the first experiment suggest that despite the presumably decreased activity of the AHAS enzyme caused by valine inhibition resulting in less diacetyl formed during fermentation, the AHAS enzyme can still actively catalyse the formation of α-acetohydroxybutyrate from α-ketobutyrate, since the concentrations of 2,3-pentanedione were not significantly affected by the valine supplementations. It is unclear whether inhibition of the AHAS enzyme by valine still allows the α-ketobutyrate to α-acetohydroxybutyrate reaction to be active, and if isoleucine or the other branched-chain amino acids have any inhibiting effect on the α-ketobutyrate to α-acetohydroxybutyrate reaction. Results from the study by Epelbaum *et al.* (1996) on the effect of sulfometuron methyl on the activity of enzymes in the valine and isoleucine synthesis pathways in *Salmonella typhimurium*, suggest that sulfometuron methyl only inhibits the pyruvate to α-acetolactate reaction of the AHAS enzyme, while the α-ketobutyrate to α-acetohydroxybutyrate reaction remains active. Since studies on the AHAS activity (e.g. Byrne and Meacock, 2001; Duong *et al.*, 2011; Magee and de Robichon-Szulmajster, 1968; Pang and Duggleby, 2001) revolve around an assay based on the ability of AHAS to convert pyruvate into α-acetolactate, it would be of interest to develop a method for measuring the activity of the α-ketobutyrate to α-acetohydroxybutyrate reaction as well, and the effect of various amino acids on its activity.

If inhibition by isoleucine of the AHAS enzyme is not the cause for the lowered 2,3-pentanedione concentrations observed during fermentation of the NPAA- and BCAA-supplemented worts, both containing isoleucine, another explanation is that the threonine deaminase enzyme, responsible for catalysing the deamination of threonine to α-ketobutyrate in the first step of the isoleucine synthesis pathway, is induced by valine and inhibited by isoleucine (Katsunuma *et al.*, 1971). This suggests that less α-ketobutyrate, and consequently less α-acetohydroxybutyrate and 2,3-pentanedione, would be formed during fermentation of the NPAA- and BCAA-supplemented worts. This could also explain the slightly larger difference in

maximum 2,3-pentanedione concentration compared to diacetyl concentration between the PAA-supplemented and control wort, since the PAA group contained threonine, most likely resulting in more α-ketobutyrate being synthesized. The amount of diacetyl produced during fermentation could also potentially be decreased by increasing the concentrations of wort threonine, as it would increase the amount of α-ketobutyrate formed via threonine deaminase, which would then compete with pyruvate for the active site of the AHAS enzyme, resulting in less formation of α-acetolactate. This would though result in higher concentrations of 2,3-pentanedione during fermentation, but since it has a 10-fold flavour threshold compared to diacetyl the effect on beer quality would be minor. Threonine is also absorbed rapidly into the yeast cells, thus increased threonine concentrations wouldn't have a significant impact on valine uptake.

The results from the third experiment imply that modifying the concentrations of wort amino acids has a slight effect on the concentrations of aroma compounds in the beer. The concentrations of higher alcohols, and esters derived from these alcohols, produced in the yeast via the transamination of amino acids were increased in almost all cases when the concentration of the relevant amino acid precursor was increased, suggesting a positive correlation especially between the uptake rate of branched-chain amino acids and the higher alcohols produced from them. The concentrations of the higher alcohols remained below or around the flavour threshold though in all cases (Meilgaard, 1982; Siebert, 1988), suggesting that any changes in higher alcohol concentrations caused by altering wort amino acid concentrations on beer quality will be minor. The concentration of 3-methylbutylacetate was however above the flavour threshold for all fermentations, even the controls, so large changes in wort leucine concentration could affect the flavour impact of this ester on the beer. The concentrations of the examined ethyl esters were around or slightly below their flavour threshold in all fermentations, and the concentrations either remained unaffected or even decreased with the supplementation of amino acids. Ester-derived flavours and aroma are only desired in small amounts in lager beers (Verstrepen *et al.*, 2003), so the minor impact of amino acid supplementation on ester concentrations is a positive result.

According to the results of the fourth experiment, there was only a small difference in diacetyl concentration between the matured beers and the concentrations were all around the flavour threshold of 100 ppb, suggesting that there was no added benefit, in regards to decreased maturation time, with the supplemented amount of valine and the maturation time used (5 days at 12° C and 2 days at 0° C). But, since there was a difference in concentrations at the end of fermentation (165 hours) and there was an even larger difference with increased supplementation levels (results from the first experiment), a shorter maturation time, increased valine supplementation, or different wort amino acid composition would most likely have resulted in a greater difference between diacetyl concentrations in the beer. Also, since fermentation rate was faster with the pre-conditioned yeast (80% apparent attenuation was reached around 20 hours faster), but the diacetyl concentrations at 165 hours were simultaneously higher than in the beers fermented with the control yeasts, valine supplementation allowed for successfully counter-acting the disadvantage of increased diacetyl production resulting from the faster fermentation rate. Foam stability was not affected by either valine supplementation or the pre-conditioning of yeast, suggesting that visual quality of the beer is not affected.

The analysis was performed with validated methods and the results were of sufficient accuracy to draw reliable conclusions. All fermentations were performed in duplicate, and the results of analysis (with the exception of amino acid concentration and certain vicinal diketone concentration analysis) were presented as averages from the duplicates with error bars spanning the standard deviation of the measurements. Measurements of amino acid concentrations were only performed on a single fermentation from each pair of duplicates, and hence their uncertainty is higher. The relative standard deviation of the amino acid concentrations was though below 2.2% for all measurements. Measurements of vicinal diketone concentrations were done on a single fermentation from each pair of duplicates in the first and third experiment, while on both fermentations of each pair of duplicates in the second and fourth experiment. The analysis of vicinal diketone concentrations was performed in triplicate, and any standard deviation between measurements was factored in to the potential standard deviation between the duplicate fermentations according to Tatebe

(2005). As can be seen from the results, there was little variance between the duplicate fermentations in terms of fermentation performance (alcohol content, yeast mass and pH). The analysis was performed using standard methods published by the European Brewery Convention (2008) or with standard protocols at VTT, so the methods have been proven to provide sufficient accuracy for the measured variables. Potential errors and inaccuracy of the results may also have resulted from volume measurement errors during the use of small sample volumes, differences in amount of yeast pitched (as a result of uneven distribution of yeast in slurries caused by sedimentation), and from errors occurring under sample preparation, e.g. evaporation of the volatile vicinal diketones and aroma compounds or continued metabolic activity as a result of insufficient separation of yeast from the samples.

6 Conclusions

The objective of this thesis was to investigate the influence of valine supplementation, wort amino acid profile and free amino nitrogen content on the production of diacetyl during wort fermentation with the lager yeast *S. pastorianus*, and this was accomplished successfully through a series of small-scale and medium-scale fermentations. The results from all four experiments agree in that the diacetyl concentration of fermenting wort can be decreased by modifying its initial amino acid profile, and particularly the concentrations of valine and the other branched-chain amino acids, without any significant effect on fermentation performance. Consequently, the maturation time of the beer can potentially be decreased as well, resulting in potential cost savings for breweries. The results from the experimental work suggest that the uptake rate of amino acids and their intracellular effect on the metabolic flux through the valine and isoleucine biosynthesis pathway are vital for understanding their relationship with diacetyl and 2,3-pentanedione production. The optimal amino acid composition of wort, from a low-diacetyl perspective, would contain high fractions of valine, leucine and potentially threonine, low fractions of non-preferred amino acids defined in this thesis, and the overall FAN content would be low enough to not effect yeast growth. Among the vast range of different strategies for diacetyl reduction (see Section 2.3.3), the majority of the recent ones rely on the use of GM strains, which currently are unsuitable for industrial use. Hence, further research into modifying the wort amino acid profile could yield valuable techniques for reducing diacetyl without the use of GM strains.

To further identify the relations between different amino acids and the metabolic activity of the valine and isoleucine biosynthesis pathway, future research could involve examining the effects of individual amino acids on both the activity of and expression of the genes encoding and the various enzymes (e.g. AHAS) and transporter proteins (e.g. Bap2p and Bap3p) involved. This would allow for the modelling of an optimal amino acid composition from a low diacetyl perspective. To pinpoint the individual effects of leucine and isoleucine, fermentations could also be performed supplementing various amounts of them and examining the effect on

diacetyl concentration, as was done in the first experiment with valine, and the fermentations of the third experiment could be repeated by leaving out leucine and isoleucine from the PAA and NPAA groups respectively, to examine if any effect on valine uptake and diacetyl production was actually caused by leucine and isoleucine rather than increase in concentrations of the other amino acids in the groups. The experiments conducted during this thesis involved specific fermentation conditions (constant temperature, pitching rate, yeast strain, and oxygenation amount), so fermentations could be repeated with different process variables to examine their effect on diacetyl production and valine uptake as well. Further research in malting and mashing technology (e.g. targeted proteolysis) could also be performed, to investigate and develop methods for optimizing the amino acid profile of the wort, to decrease diacetyl production during fermentation. Charting of the amino acid composition of various brewery side-streams, cereal grains, and high-protein solutions could also yield knowledge on economical sources of amino acids adjuncts with a favourable amino acid composition, since direct supplementation of purified valine to brewer's wort is most likely not economically viable compared to the costs of saved maturation time. To find yeast strains with a naturally lower diacetyl production rate and higher valine uptake rate, yeast strains could be screened by growing them on media supplemented with AHAS inhibitors (e.g. sulfometuron methyl (Gjermansen *et al.*, 1988)), as any growth would indicate either a resistant enzyme or high uptake rates of the branched-chain amino acids (since their biosynthesis is hindered). Hence, it can be concluded that despite the clear results from the experiments conducted during this thesis, there are still plenty of potential areas for further research that need to be covered before more accurate relationships between amino acids and diacetyl production are established, and the techniques can be implemented on an industrial scale.

7 References

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Specifications of all-malt worts **Appendix 1**

Table 1 – Specifications of Wort 732.

Parameter	**Value**
Original Extract	15.0° Plato
pH	5.17
FAN	407.6 mg/L
EBU	37
Apparent Attenuation Limit	86.4%

Table 2 – Specifications of Wort 735.

Parameter	**Value**
Original Extract	15.0° Plato
pH	5.17
FAN	362.9 mg/L
EBU	36
Apparent Attenuation Limit	Not determined

Table 3 – Specifications of Wort 737.

Parameter	**Value**
Original Extract	15.0° Plato
pH	5.16
FAN	362.2 mg/L
EBU	34
Apparent Attenuation Limit	Not determined

Amino acid absorption rates in yeast **Appendix 2**

Table 1 – The average linear change in amino acid concentrations during the first 25 hours of fermentation of all-malt wort 732 with the *Saccharomyces pastorianus* strain A15.

Amino acid	**Decrease in concentration ($\mu mol \cdot (l \cdot min)^{-1}$)**
Asparagine	0.575
Serine	0.465
Threonine	0.382
Leucine	0.368
Lysine	0.276
Proline	0.173
Arginine	0.161
Phenylalanine	0.124
Glutamine	0.107
Aspartic acid	0.106
Valine	0.104
Isoleucine	0.104
Histidine	0.059
Glycine	0.053
Glutamic acid	0.039
Tryptophan	0.007
Alanine	-0.012
Methionine	-0.030
Tyrosine	-0.046

The pH of worts during experimental fermentations **Appendix 3 (1/2)**

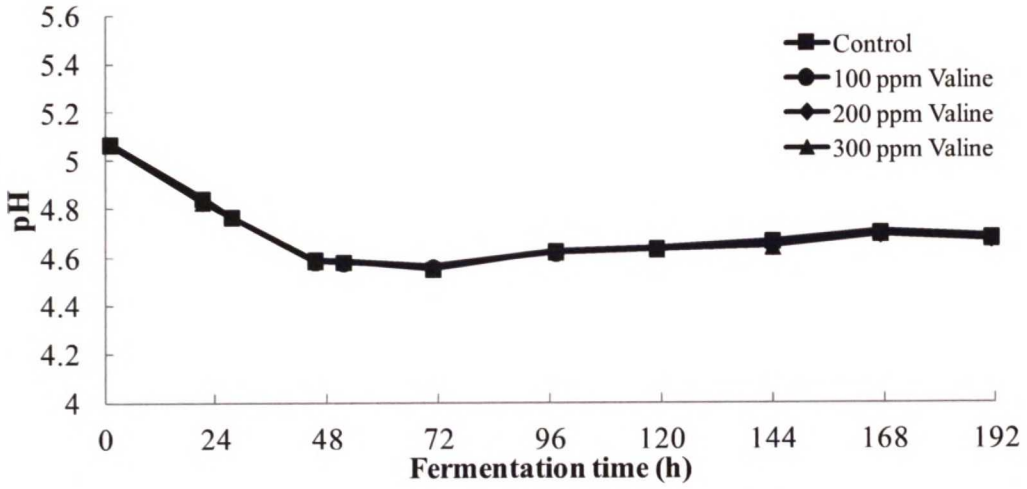

Figure 1 – The pH of the valine-supplemented worts used for the experimental fermentations of the first experiment as a function of fermentation time (hours). Values are means from two independent fermentations. Error bars where visible represent standard deviation of mean.

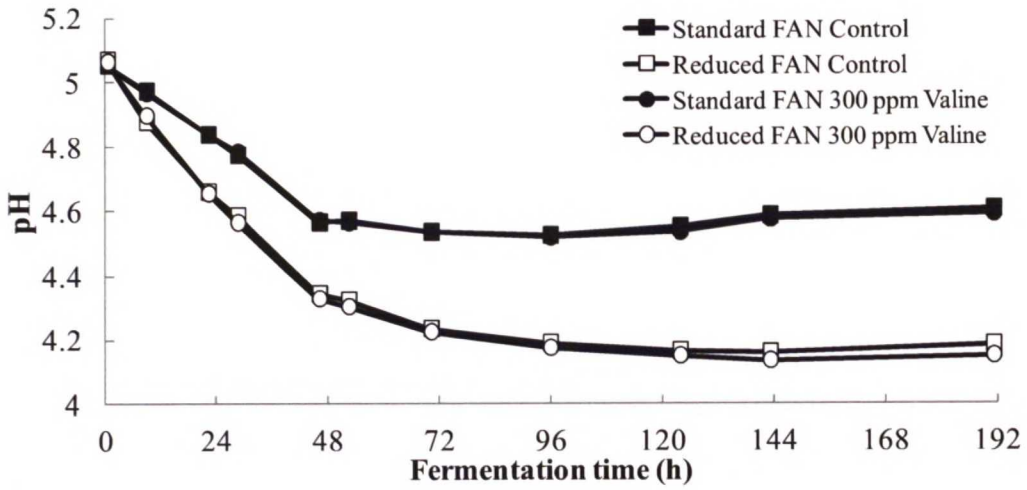

Figure 2 – The pH of the valine-supplemented of the worts used for the experimental fermentations of the second experiment as a function of fermentation time (hours). Values are means from two independent fermentations. Error bars where visible represent standard deviation of mean.

The pH of worts during experimental fermentations **Appendix 3 (2/2)**

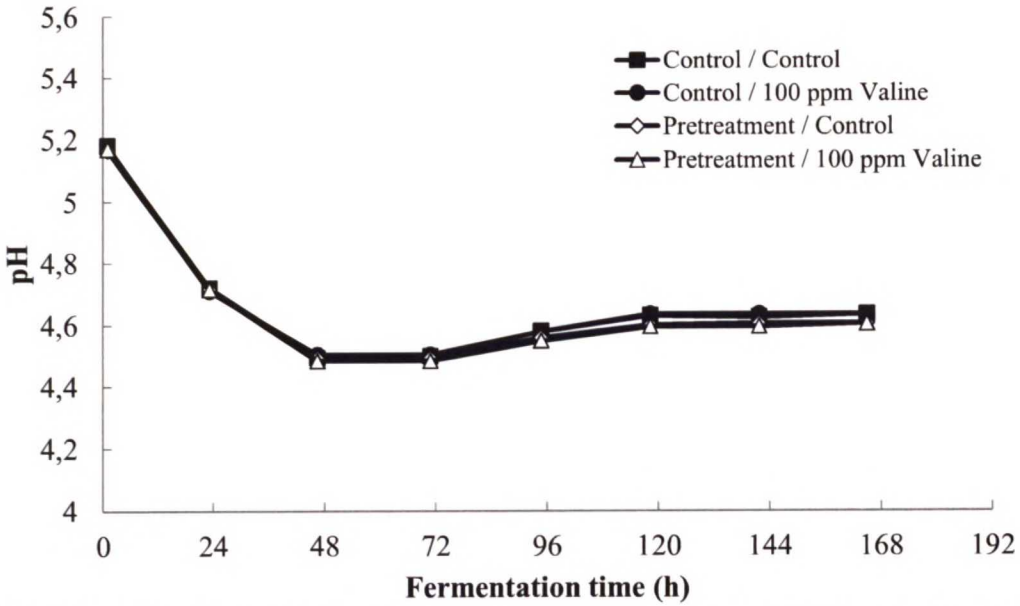

Figure 3 – The pH of the valine-supplemented of the worts used for the experimental fermentations of the fourth experiment as a function of fermentation time (hours). Values are means from two independent fermentations. Error bars where visible represent standard deviation of mean.