Appl Microbiol Biotechnol (2008) 80:1039–1051
DOI 10.1007/s00253-008-1645-5

Monitoring the influence of high-gravity brewing and fermentation temperature on flavour formation by analysis of gene expression levels in brewing yeast

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Received: 19 June 2008 / Revised: 30 July 2008 / Accepted: 2 August 2008 / Published online: 27 August 2008

Abstract During fermentation, the yeast *Saccharomyces cerevisiae* produces a broad range of aroma-active substances, which are vital for the complex flavour of beer. In order to obtain insight into the influence of high-gravity brewing and fermentation temperature on flavour formation, we analysed flavour production and the expression level of ten genes (*ADH1*, *BAP2*, *BAT1*, *BAT2*, *ILV5*, *ATF1*, *ATF2*, *IAH1*, *EHT1* and *EEB1*) during fermentation of a lager and an ale yeast. Higher initial wort gravity increased acetate ester production, while the influence of higher fermentation temperature on aroma compound production was rather limited. In addition, there is a good correlation between flavour production and the expression level of specific genes involved in the biosynthesis of aroma compounds. We conclude that yeasts with desired amounts of esters and higher alcohols, in accordance with specific consumer preferences, may be identified based on the expression level of flavour biosynthesis genes. Moreover, these results demonstrate that the initial wort density can determine the final concentration of important volatile aroma compounds, thereby allowing beneficial adaptation of the flavour of beer.

Keywords Flavour · Ester · Higher alcohol · Fermentation · Yeast

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Introduction

The organoleptic characteristics of beer depend mainly on the aroma-active substances produced by yeast during the fermentation of wort. An important consideration in beer fermentation and flavour maturation is the fine-tuning of the concentration of all volatile compounds. However, the absolute amount of flavour-active compounds is not really relevant by itself for beer flavour. More important is the relationship between the concentration and the sensory flavour threshold of the individual compounds. In spite of this, a flavour compound, present below its threshold, can have an impact on the beer aroma through synergy with other flavour compounds present in the beer (matrix effect) (Meilgaard 1975a).

While most of today's lager and ale beers are produced by high-gravity brewing, it is well known that the fermentation of wort of high specific gravity leads to the relative overproduction of acetate esters. This results in over-fruity and solvent-like beers (Anderson and Kirsop 1974). Another important fermentation variable is the temperature. A higher temperature increases the fermentation rate and the final concentration of higher alcohols (Landaud et al. 2001) and esters (Saerens et al. 2008).

Of all secondary metabolites, higher alcohols are generally produced by yeast in the highest absolute concentrations.Those of importance for beer aroma are: propanol (solvent-like),

isobutanol (alcoholic), isoamyl alcohol (fruity, sweet) and 2-phenyl ethanol (rose, floral) (Meilgaard 1975b; Quain and Duffield 1985). Biosynthesis of higher alcohols involves the decarboxylation of α-keto-acids to form aldehydes, followed by a reduction of the aldehydes to produce the corresponding alcohols (Dickinson et al. 1997). The α-keto-acids are formed via two major pathways: the catabolic Erhlich pathway, involving degradation of amino acids to their corresponding alcohols and an anabolic pathway, involving de novo synthesis of branched-chain amino acids from glucose. The first and rate-limiting step in the catabolism of branched-chain amino acids is catalysed by mitochondrial and cytosolic branched-chain amino acid aminotransferases encoded by *BAT1* and *BAT2*, respectively (Eden et al. 1996, 2001). Overexpression of *BAT1* increases the concentration of isoamyl alcohol, isoamyl acetate and, to a lesser extent, isobutanol (Lilly 2004). The uptake of branched-chain amino acids by *S. cerevisiae* is mediated by the *BAP2*-encoded branched-chain amino acid permease (Didion et al. 1996). Constitutive expression of the *BAP2* gene results in increased production of isoamyl alcohol, but no increase in isobutanol or amyl alcohol production (Kodama et al. 2001).

The production of higher alcohols is directly related to amino acid metabolism, which is in turn related to cell growth (Kruger 1998; Quain and Duffield 1985). This explains why wort oxygenation, high fermentation temperatures, and unsaturated fatty acids and sterols cause an increase in higher alcohol production (Quain and Duffield 1985; Valero et al. 2002). High fermentable sugar levels, as found in high-gravity brewing, also lead to increases in the production of certain higher alcohols (Engan 1972; Szlavko 1974; Younis and Stewart 1999). The synthesis of 2-phenyl ethanol is extremely sensitive to changes in fermentation temperature.

Although volatile esters are only present in trace quantities in beer, they are extremely important for the flavour profile (Verstrepen et al. 2003b,c). The most important flavour-active esters are ethyl acetate (solvent-like), isoamyl acetate (banana), ethyl hexanoate and octanoate (sour apple), and phenyl ethyl acetate (roses, honey) (Verstrepen et al. 2003b). In lager and ale beers, the concentration of isoamyl acetate and ethyl hexanoate reaches the threshold levels of detection. However, the presence of different esters can have a synergistic effect on the individual flavours. As most esters are present in concentrations around their threshold value, minor changes in their concentration may have dramatic effects on beer flavour (Meilgaard 1975a).

Volatile esters are the product of an enzyme-catalysed condensation reaction between an acyl-CoA and a higher alcohol, catalysed by alcohol acetyltransferases, which are encoded by the genes *ATF1* and *ATF2*. The expression levels of *ATF1* and *ATF2* greatly affect the production of ethyl and isoamyl acetate (Verstrepen et al. 2003c). In addition to these alcohol acetyltransferases, two other enzymes, encoded by *EHT1* and *EEB1*, have been described as being responsible for the production of ethyl esters (Mason and Dufour 2000; Saerens et al. 2006). Eeb1 is the main enzyme, while Eht1 plays only a minor role. Furthermore, the balance between ester-synthesising enzymes and esterases, which hydrolyse esters, may be important for the net rate of ester accumulation (Fukuda et al. 1998).

Although the ester profile is highly strain dependent, the regulation of the ester levels by environmental variables shows consistent trends for many strains. This is true for the increase in acetate ester levels in high-gravity brewing (Younis and Stewart 1999), the decrease in ester levels with stronger oxygenation and higher unsaturated fatty acid levels in the wort (Saerens et al. 2008; Thurston et al. 1982) and the increase in the levels of esters, except ethyl hexanoate, with higher fermentation temperatures (Saerens et al. 2008).

Vicinal diketones (VDKs) are normal products of beer fermentation, but are generally considered to be undesirable contributors to the beer flavour (Inoue 1992; Wainwright 1973). The two major VDKs of concern to the brewer are diacetyl (2,3-butanedione) and 2,3-pentanedione. Diacetyl confers a butterscotch-like aroma and pentanedione a honey-like aroma. The immediate precursor of diacetyl is α-acetolactate. α-Acetolactate leaks from the cell into the fermenting medium, where it is spontaneously oxidatively decarboxylated to diacetyl. The yeast cell, however, is capable of reducing these VDKs with the intermediate product being acetoin, which may be further reduced to 2,3-butanediol.

The aim of this study was to investigate which of the known flavour biosynthesis genes are important for the production of the main yeast flavours during fermentation. For that purpose, we have analysed gene expression under different conditions. We focussed on high-gravity brewing and higher fermentation temperature since these are often used in brewing practice to increase the volumetric productivity of the brewery. Volatile compounds were quantified at different stages of the fermentations and compared to the expression level of ten genes involved in aroma biosynthesis pathways. A lager and an ale brewing strain were used for this study because the contribution of high-gravity brewing and fermentation temperature to flavour formation is an important issue in industrial production of both lager and ale beers. In this paper, we provide new evidence for the importance of specific aroma biosynthesis genes for the production of adequate quantities of the volatile aroma compounds in beer fermentation.

Materials and methods

Yeast strain and cultivating conditions All experiments were carried out with an industrial lager brewing strain (CMBS SS03) or an industrial ale brewing strain (CMBS SS10; Katholieke Universiteit Leuven, Centre for Malting and

Brewing Science, Heverlee, Belgium). Industrial propagated yeast was used for prefermentation in a pilot-scale brewery. After 5 days of fermentation in 12 degrees Plato (°P; g extract/100 g wort) all-malt hopped wort, yeast slurry was taken from the bottom of the fermentation tank. The viable yeast slurry was quantified by flow cytometry (YeastCyte, *Bio*DETECT AS, Oslo, Norway) before the required amount was pitched in the wort. The yeast was inoculated at a pitching rate of 20×10^6 viable cells/ml for the lager strain and 6×10^6 viable cells/ml for the ale strain.

Fermentation conditions Brewers report wort strengths in degrees Plato (°P; g extract/100 g wort). Sterile all-malt hopped wort (16°P; 66% maltose, 15% maltotriose, 16% glucose, 3% fructose) with a free amino nitrogen (FAN) content of 288 ppm was made in a pilot brewery and used for the fermentation with the lager strain. Twelve degrees Plato wort was prepared by dilution of the 16°P wort (66% maltose, 15% maltotriose, 17% glucose and 2% fructose) and had a FAN content of 234 ppm. Sterile all-malt hopped wort (18°P) (68% maltose, 18% maltotriose, 13% glucose, 1% fructose) with a FAN content of 415 ppm was made in a pilot brewery and used for the fermentation with the ale strain. 14°P wort was prepared by dilution of the 18°P wort (68% maltose, 17% maltotriose, 14% glucose and 1% fructose) and had a FAN content of 334 ppm.

All fermentations were carried out in duplicate, in tall tubes, containing 1.8 l sterile wort medium. The wort was aerated by filling the headspace with sterile air followed by intensive mixing by inverting the tubes twenty times. The fermentations were performed at 12°C or 15°C for the lager strain and 20°C and 24°C for the ale strain and were monitored daily.

Fermentation analysis The course of fermentation and yeast growth was followed by withdrawing samples anaerobically, cooling them on ice and separating the yeast and fermenting wort by centrifugation. Before centrifugation, the number of suspended yeast cells was counted by flow cytometry (YeastCyte, *Bio*DETECT AS, Oslo, Norway). The specific gravity of the fermenting medium was measured with a handheld density meter (DMA 35N, Anton Paar, Graz, Austria), and the final extract and alcohol content were measured with the DMA 4500 density analyser and Alcoholyser Plus (Anton Paar, Graz, Austria). FAN was determined by a ninhydrin-based standard method as defined by the European Brewery Convention (EBC Analytica, 1998). Glucose concentrations were determined by a D-glucose-kit (D-glucose GOPOD format, Megazyme International, Ireland).

Headspace GC analysis Headspace gas chromatography (HS-GC) coupled with flame ionisation detection (GC-FID) was used for the measurement of acetate esters, ethyl esters and higher alcohols in the fermentation products. Headspace gas chromatography coupled with electron capture detection (GC-ECD) was used for the measurement of vicinal diketones in the fermentation products. Fermentation samples were cooled on ice, centrifuged and filtered, after which 5 ml was collected in vials, which were immediately closed. Before analysis, the samples were heated at 60°C for 1 h to cause complete conversion of α-acetolactate into diacetyl. Samples were then analysed with a calibrated Autosystem XL gas chromatograph with a headspace sampler (HS40; Perkin-Elmer, Wellesley, MA, USA) and equipped with a CP-Wax 52 CB column (length, 50 m; internal diameter, 0.32 mm; layer thickness, 1.2 μm; Chrompack, Varian, Palo Alto, CA, USA). Samples were heated for 25 min at 70°C in the headspace autosampler before injection (needle temperature: 105°C). Helium was used as the carrier gas. The oven temperature was held at 50°C for 5 min, then increased to 200°C at a rate of 5°C per min and finally held at 200°C for 3 min. The FID and ECD temperatures were kept constant at 250°C and 200°C, respectively. Analyses were carried out in duplicate, and the results were analysed with Perkin-Elmer Turbochrom Navigator software. To determine the end values of the aroma compounds, the results were recalculated to 5.1% (*v*/*v*) ethanol to normalise for the effect of temperature and specific gravity on final ethanol level (Table 2).

Quantitative PCR The expression levels of *ATF1*, *ATF2*, *IAH1*, *EHT1*, *EEB1*, *ADH1*, *BAT1*, *BAT2*, *BAP2* and *ILV5* were determined using quantitative-PCR (qPCR). Fifty-milliliter samples were collected from the tall tubes and centrifuged at 3,000 rpm for 5 min. The pelleted cells were then frozen at –80°C. RNA extraction of 100×10^6 pelleted cells was performed with Trizol (Invitrogen), according to the manufacturer's instructions. For each sample, 1 μg of total RNA was subject to reverse transcription (RT) using the Reverse Transcription System (Promega A3500, Madison, WI, USA). Concentrations were measured and samples diluted to 70 ng μl^{-1}. The 25 μl PCR reaction was composed of 12.5 μl Platinum SYBR Green qPCR SuperMix-UDG with ROX (Applied Biosystems) and 1.25 μl of each primer (500 nM). Five microliters of cDNA was added to each reaction mix. The PCR program used consisted of an initial denaturation for 10 min at 95°C, amplification by 40 cycles of 15 s at 95°C and 1 min at the optimal annealing temperature for the specific primer pair. The annealing temperature was optimised for each primer pair under qPCR conditions: 58°C for *RDN18* F-R, *ATF1* F-R, *ATF2* F-R, *IAH1* F-R, *ADH1* F-R, *BAT1* F-R, *BAT2* F-R, *BAP2* F-R and *ILV5* F-R, and 56°C for *EHT1* F-R and *EEB1* F-R. The reverse primer was designed to anneal close to the 3′-end of each gene. The PCR primers were all designed with the PRIMER EXPRESS software (PE Applied Biosystems, Cheshire, UK) according to the PE Applied Biosystems guidelines. Primer sequences

used for qPCR analysis (from 5′ to 3′): *RDN18*-F: CGGCT ACCACATCCAAGGAA, *RDN18*-R: GCTGGAATTACC GCGGCT, *ATF1*-F: GTACGAGGAGGATTACCA, *ATF1*-R: ATGATCTCGGTGACAAC, *ATF2*-F: AAGCCGTACT ACGTTCG, *ATF2*-R: CGCT CATGTCCATGTTC, *IAH1*-F: TCCGTACCAACGAGAAC, *IAH1*-R: TTGCCAAG CAT CACCAC, *EHT1*-F: TGGCTCTCCCCGATCA, *EHT1*-R: AGGCGTGAACA TATAGAAAGATGGA, *EEB1*-F: TCGTACACACTTGGGACAAGTTG, *EEB1*-R: CAGTCCTTGTTAGAAATTGTGTTAAAGTTC, *ADH1*-F: TCACGCTGACTTGTC TG, ADH1-R: GCCTTGTAGAC GGTGAT, *BAT1*-F: CCAAATCCATCCAAGCCA AG, *BAT1*-R: AGCAGATGGGTCAAGA GAAA, *BAT2*-F: CCGGTCCATTGGCCA AA, *BAT2*-R: ACCAATTGCCAT GCTCAGTCT, *BAP2*-F: TGGTTGGCCTTTTACT TCGGA, *BAP2*-R: TTCGTCCTCTTGTCTCATTAG, *ILV5*-F: ACGGTGAAA GAG GTTGTTTA, *ILV5*-R: CCGATCAATGGGTATAGAGA. The expression levels were analysed with ABI prism (PE Applied Biosystems, Cheshire, UK). The gene for 18S rRNA (*RDN18*) was used as the reference gene because its expression was found to be stable under our experimental conditions. The expression level of the different genes was normalised with respect to the 18S expression level.

Results

The industrial lager yeast CMBS SS03 and ale yeast CMBS SS10 were used to evaluate the influence of high-gravity brewing and fermentation temperature on flavour formation, in relation to specific gene expression. Fermentations were carried out in EBC tall tubes under anaerobic conditions at 12°C and 15°C for the lager yeast and 20°C and 24°C for the ale yeast. The tall-tubes contained 1.8 l sterile all malt-hopped wort medium of 16°P or 12°P for the lager yeast and 18°P or 14°P for the ale yeast.

Fermentation performance

Three conditions were used for the tall-tube fermentations: low density and low temperature (12°P and 12°C for the lager strain and 14°P and 20°C for the ale strain), low density and high temperature (12°P and 15°C for the lager strain and 14°P and 24°C for the ale strain) and high density and high temperature (16°P and 15°C for the lager strain and 18°P and 24°C for the ale strain). The progress of the fermentations is shown in Fig. 1. All the fermentations proceeded normally. The fermentations were stopped when the apparent degree of attenuation reached 80% (Table 1). FAN amounts were de-

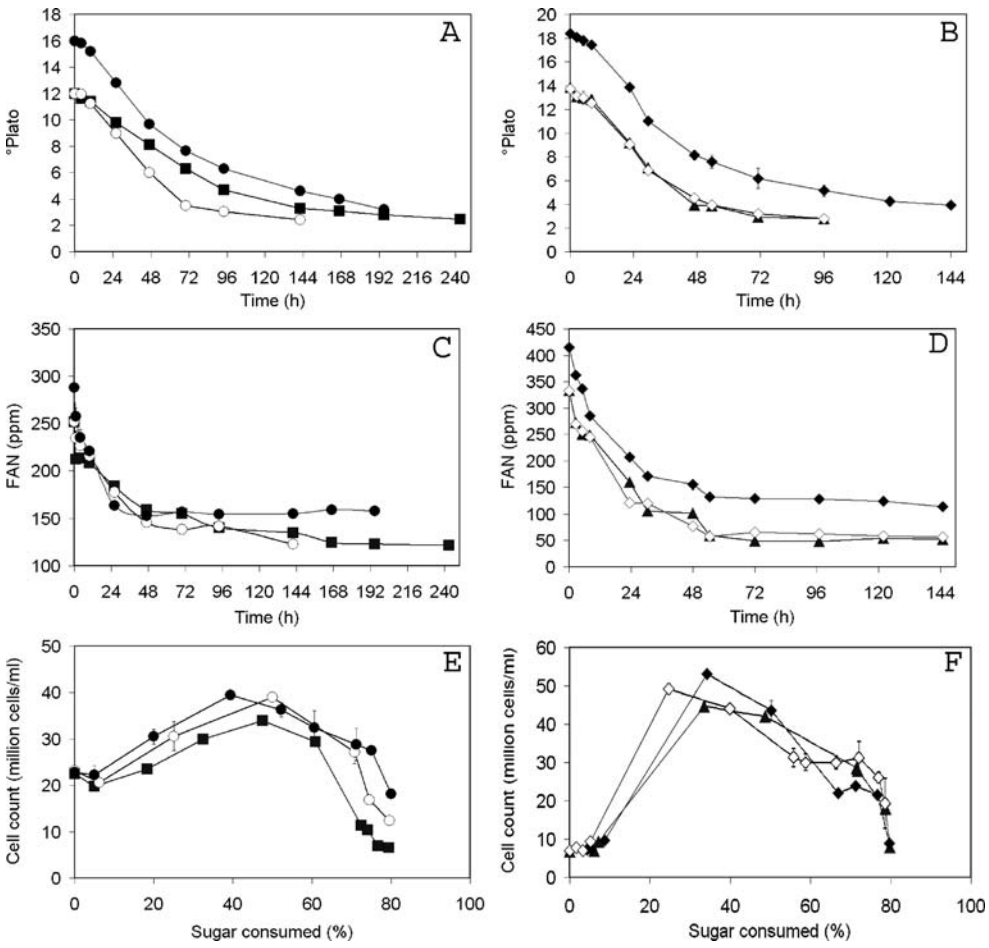

Fig. 1 Fermentation profile of tall tube fermentations with a lager (**a**, **c** and **e**) and an ale (**b**, **d** and **f**) brewing strain. Course of sugar content (expressed as °Plato; **a** and **b**), free amino nitrogen (FAN) content (**c** and **d**) and cell density (**e** and **f**) throughout the fermentations. Results are the averages of two independent fermentations. *filled squares*: 12°P 12°C, *empty circles*: 12°P 15°C, *filled circles*: 16°P 15°C, *filled triangles*: 14°P 20°C, *empty diamonds*: 14°P 24°C and *filled diamonds*: 18°P 24°C

Table 1 Final fermentation values

Parameter	End extract (°P)	Attenuation percentage	Final ethanol conc (% v/v)	pH
12°P 12°C	2.46°P±0.02	79.50%±0.00	5.14%±0.01	4.80±0.00
12°P 15°C	2.45°P±0.07	79.58%±0.59	5.50%±0.03	4.52±0.01
16°P 15°C	3.20°P±0.01	80.00%±0.00	6.41%±0.13	4.63±0.04
14°P 20°C	2.80°P±0.00	79.78%±0.00	5.14%±0.00	4.17±0.01
14°P 24°C	2.80°P±0.01	79.64%±0.00	6.41%±0.13	4.13±0.01
18°P 24°C	3.95°P±0.07	78.53%±0.38	5.47%±0.10	4.25±0.02

termined throughout the fermentation, which showed that FAN consumption was higher for the ale yeast than the lager yeast, but similar in all conditions tested (Fig. 1c and d). A higher pH for the fermentation with the lager yeast compared to the fermentation with the ale yeast was observed, which could mean that the lager yeast cells were beginning to autolyse at the end of fermentation.

As some fermentations proceeded faster, time was not an appropriate parameter to compare the progress of the fermentation. Therefore, the data were normalised with respect to the relative sugar content, i.e., the percentage of sugar consumed relative to the initial sugar content, representing the physiological state of the cell at a given moment. Using this parameter, cell density evolution during the fermentations was similar for all fermentations (Fig. 1e and f). However, maximum cell density was slightly lower for the fermentations carried out at low density and low temperature. This means that the net yeast growth (the maximum yeast cell count minus the initial inoculum size) of the yeast cell population in those fermentations was slightly lower than the other fermentations. The exponential growth phase started at 95% of the initial sugar content and ended when about 40% (ale strain) or 50% (lager strain) of the sugar had been consumed. At that point, the cell density in suspension started to decrease strongly because of flocculation of the yeast.

Flavour formation

During the fermentations, the production of twelve volatile compounds was analysed by HS-GC. The results of the HS-GC analysis are shown in Table 2. The CMBS SS03 and CMBS SS10 strains synthesised a variety of flavour com-

Table 2 Final aroma concentration in fermentations performed with the lager yeast (12°P 12°C, 12°P 15°C and 16°P 15°C) and the ale yeast (14°P 20°C, 14°P 24°C or 18°P 24°C), determined by head-space GC-FID and head-space GC-ECD

Aroma compound	12°P 12°C (mg/l)±SD	12°P 15°C (mg/l)±SD	16°P 15°C (mg/l)±SD	14°P 20°C (mg/l)±SD	14°P 24°C (mg/l)±SD	18°P 24°C (mg/l)±SD	Threshold (mg/l)
Alcohols							
Propanol	11.37±0.10	12.82±0.28	13.43±0.30	30.95±1.09	27.32±0.02	42.02±0.00	600[b]
Isobutanol	7.13±0.13	7.55±0.07	8.20±0.12	35.07±0.09	33.78±1.28	37.75±0.99	100[b]
Isoamyl alcohol	58.29±0.59	59.98±1.25	58.08±0.18	126.66±1.26	116.6±0.50	123.15±0.93	50[b]
Phenyl ethanol	25.37±1.10	28.11±1.15	27.18±1.13	64.82±0.35	60.96±1.13	66.16±1.65	40[b]
Total alcohols	102.16±1.92	108.46±2.76	106.88±1.72	257.50±2.79	238.42±2.93	269.08±3.57	
Acetate esters							
Ethyl acetate	23.02±0.13	22.01±1.87	29.71±0.16	78.86±0.50	72.23±0.40	129.23±0.59	30[a]
Isoamyl acetate	1.20±0.01	1.73±0.07	1.84±0.00	8.50±0.07	7.98±0.03	9.32±0.02	1.2[a]
Phenyl ethyl acetate	0.59±0.01	0.66±0.01	0.73±0.01	2.43±0.09	2.85±0.04	3.38±0.04	3.8[a]
Total acetate esters	24.81±0.15	24.40±1.95	32.28±0.17	89.79±0.66	83.06±0.47	141.93±0.65	
Ethyl esters							
Ethyl hexanoate	0.154±0.002	0.186±0.008	0.232±0.012	0.210±0.007	0.209±0.019	0.254±0.001	0.21[a]
Ethyl octanoate	0.463±0.003	0.532±0.001	0.504±0.009	0.430±0.001	0.271±0.028	0.395±0.020	0.9[a]
Ethyl decanoate	0.103±0.005	0.109±0.013	0.082±0.005	0.035±0.010	0.029±0.001	0.019±0.002	1.5[a]
Total ethyl esters	0.720±0.010	0.827±0.022	0.818±0.026	0.675±0.018	0.509±0.048	0.668±0.023	
Vicinal diketones							
Diacetyl	0.332±0.002	0.267±0.008	0.144±0.003	0.109±0.001	0.066±0.002	0.049±0.001	0.15[a]
Pentanedione	0.279±0.003	0.257±0.005	0.184±0.006	0.044±0.001	0.054±0.002	0.015±0.001	0.9[a]
Total vicinal diketones	0.611±0.005	0.525±0.013	0.328±0.009	0.153±0.002	0.120±0.004	0.064±0.002	

Values have been re-calculated to an ethanol percentage of 5.1% *v/v*.
[a] Meilgaard 1975b
[b] Engan 1972

pounds, of which some were present in concentrations above their detection threshold.

Higher alcohols

All the higher alcohols measured were accumulated throughout the fermentation and reached their maximum at the end (Fig. 2). Increasing the fermentation temperature resulted in an accelerated production of propanol, isobutanol, isoamyl alcohol and phenyl ethanol by the two strains. However, when all the end values were recalculated to 5.1% ethanol for both strains (Table 2), there was only an increase of 12% for propanol and phenyl ethanol for the lager strain. For the ale strain, the increase in fermentation temperature had no effect on the end concentration of higher alcohols. However, as a higher temperature was already applied for the ale strain, it is possible that the temperature increase was too small to measure clear differences in flavour compounds.

When the fermentations with different initial wort density were compared, all higher alcohols showed an increased accumulation. However, recalculated to a final ethanol concentration of 5.1% *v/v*, no significant differences between the fermentation with low wort density and high wort density were found for the lager strain (Table 2). On the contrary, the end levels of higher alcohols produced by the ale strain were significantly higher when a higher initial wort density was

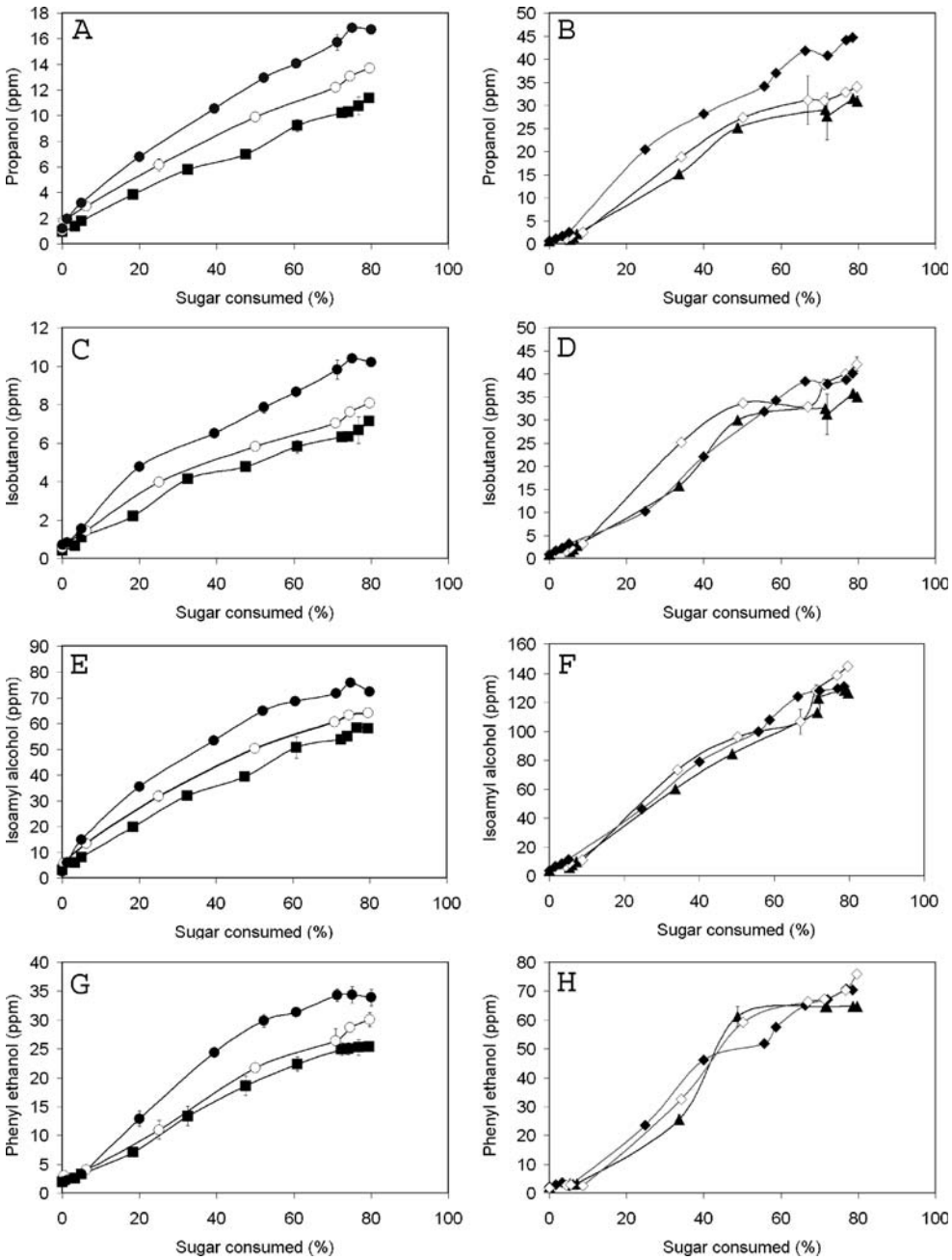

Fig. 2 Higher alcohols produced in tall tube with a lager (**a**, **c**, **e** and **g**) and an ale (**b**, **d**, **f** and **h**) brewing strain. Production of propanol (**a** and **b**), isobutanol (**c** and **d**), isoamyl alcohol (**e** and **f**) and phenyl ethanol (**g** and **h**). Results are the averages of two independent fermentations. *filled squares*: 12°P 12°C, *empty circles*: 12°P 15°C, *filled circles*: 16°P 15°C, *filled triangles*: 14°P 20°C, *empty diamonds*: 14°P 24°C and *filled diamonds*: 18°P 24°C

used. Although the threshold values for higher alcohols in beers are relatively high, isoamyl alcohol reached its threshold value in all fermentation conditions with the two strains. Phenyl ethanol exceeded its threshold level only in the fermentations with the ale strain (Table 2).

Ethyl esters

The maximum biosynthetic rates of ethyl esters were found during the first half of the exponential growth phase (Fig. 3). Ethyl hexanoate production increased until 70% of the sugars was consumed and then gradually decreased to the end of the fermentation, and this for both strains (Fig. 3a and b). For the lager strain, the production of ethyl octanoate showed a similar profile, except for the fermentation at low density (12°P) and high temperature (15°C), where the ethyl octanoate concentration increased at the end of the fermentation (Fig. 3c). The fermentation at low density (12°P) and low temperature (12°C) exhibited an ethyl octanoate peak when 30% of the sugar was consumed and decreased from that moment on, until the end of the fermentation was reached. For the ale strain, ethyl octanoate production showed a different profile. Ethyl octanoate concentration increased in two stages until 60% to 70% of the sugar was consumed and then dropped towards the end of fermentation (Fig. 3d). In the three conditions, ethyl octanoate concentration reached the same maximum level. The production profile of ethyl decanoate of the ale strain was similar, except that the maximum level was highest at high density (18°P) and high temperature (24°C) and the lowest at low density and low temperature (Fig. 3f). Ethyl decanoate production by the lager strain reached maximum concentrations when 25% of the initial sugar was consumed (mid-exponential phase), and from that point its concentration decreased (Fig. 3e). The highest ethyl decanoate peak was observed for the fermentation at high density (16°P) and high temperature (15°C). The fermentations at low density (12°P) showed similar profiles for low and high temperature.

When the fermentations at low temperature (12°C) and high temperature (15°C) at the same density (12°P) are compared, the end values (recalculated to 5.1% *v*/*v* ethanol) of all ethyl esters were significantly higher at the highest temperature for the lager strain (Table 2). This was not the case for the ale strain. A higher temperature had no influence on ethyl hexanoate and decanoate production,

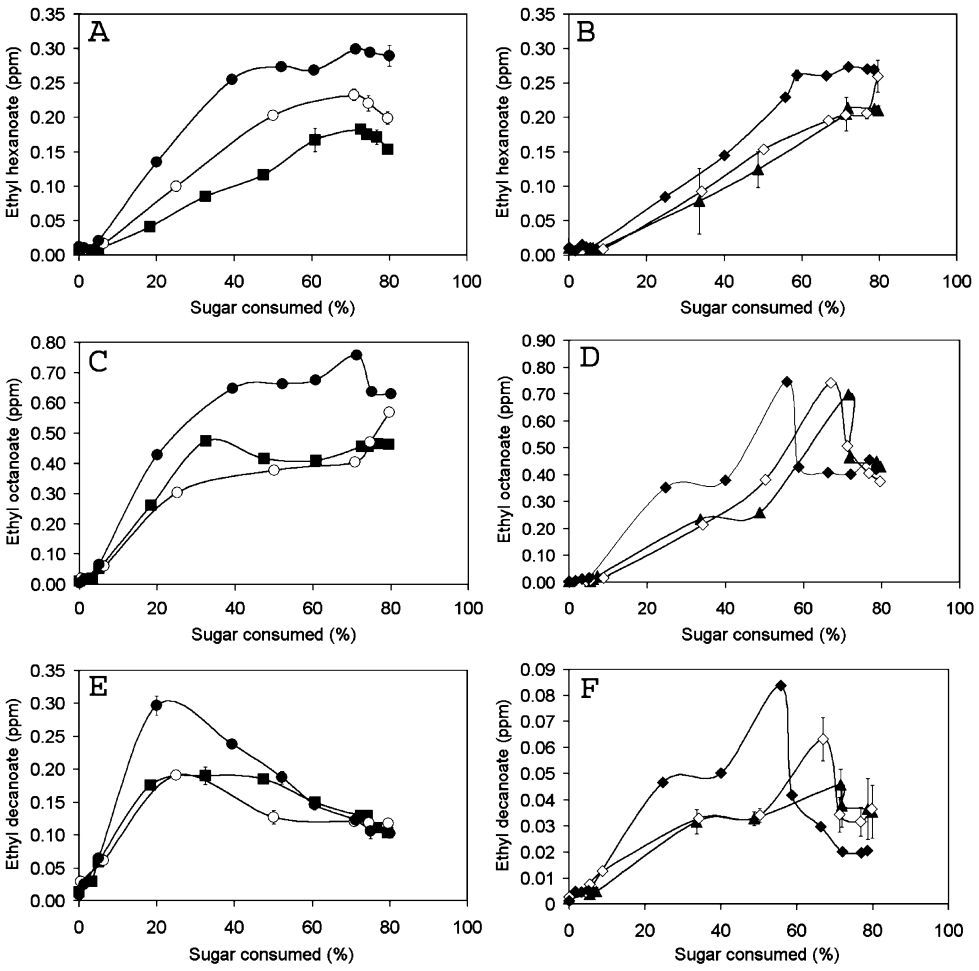

Fig. 3 Ethyl esters produced in tall tube fermentations with a lager (**a**, **c** and **e**) and an ale (**b**, **d** and **f**) brewing strain. Production of ethyl acetate (**a** and **b**), isoamyl acetate (**c** and **d**) and phenyl ethyl acetate (**e** and **f**). Results are the averages of two independent fermentations. *empty circles*: 12°P 15°C, *filled circles*: 16°P 15°C, *filled triangles*: 14°P 20°C, *empty diamonds*: 14°P 24°C and *filled diamonds*: 18°P 24°C

but caused a decrease in ethyl octanoate concentration. Again, it is possible that the temperature increase was too small for the ale strain to measure clear differences in flavour compounds.

For the fermentations at different wort densities, only the production of ethyl hexanoate was higher at the highest density for the lager strain, while both ethyl hexanoate and octanoate production increased at higher wort density for the ale strain. The concentrations of ethyl octanoate and decanoate decreased at the highest density for the lager strain. Ethyl decanoate concentration was also lower at higher density for the ale strain. Only ethyl hexanoate reached its threshold level in some conditions tested. For the lager strain, ethyl hexanoate exceeded its threshold level at high temperature and high wort density. For the ale strain, ethyl hexanoate was present around its threshold level in fermentations at low density, at both temperatures, but also exceeded its threshold level at high temperature and high wort density. As only ethyl hexanoate exceeded its threshold level, this ester is suggested to be the most important ethyl ester for flavour determination in beers.

Acetate esters

Compared to ethyl esters, acetate esters were synthesised at a slower rate during the exponential growth phase, and only reached their maximum concentration at the end of the fermentation in most cases (Fig. 4). The results shown in Table 2 (recalculated at 5.1% *v/v* ethanol) and Fig. 4a, c and e indicate that isoamyl acetate and phenyl ethyl acetate production increased with higher fermentation temperature, while ethyl acetate concentration was not affected by temperature for the lager strain. For the ale strain, the concentration of all acetate esters decreased when a higher temperature was used (Table 2).

When the fermentations with different initial wort densities are compared, all acetate ester levels were significantly higher when a higher wort density was used, and this was true for both strains. Table 2 shows that the ethyl acetate level increased with about 25% for the lager strain and 45% for the ale strain when a higher wort density was used. The concentrations of isoamyl acetate and phenyl ethyl acetate were 10% higher for the lager strain and 17% higher for the ale strain in fermentations with a higher wort density. For the lager strain,

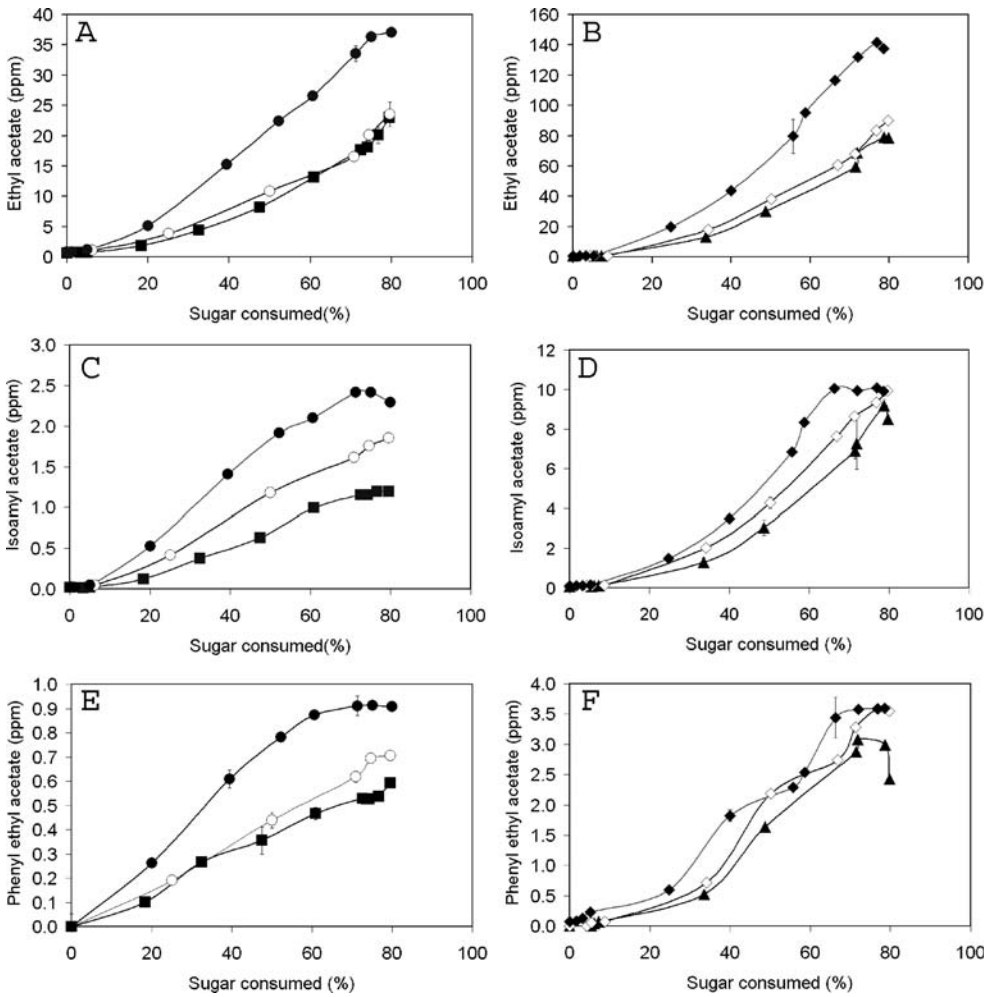

Fig. 4 Acetate esters produced in tall tube fermentations with a lager (**a**, **c** and **e**) and an ale (**b**, **d** and **f**) brewing strain. Production of ethyl hexanoate (**a** and **b**), ethyl octanoate (**c** and **d**) and ethyl decanoate (**e** and **f**). Results are the averages of two independent fermentations. *filled squares*: 12°P 12°C, *empty circles*: 12°P 15°C, *filled circles*: 16°P 15°C, *filled triangles*: 14°P 20°C, *empty diamonds*: 14°P 24°C and *filled diamonds*: 18°P 24°C

only one acetate ester, isoamyl acetate, exceeded its threshold level when a higher fermentation temperature was used. On the contrary, two of the three quantified acetate esters, ethyl acetate and isoamyl acetate, reached their threshold levels when the initial wort density was increased. Ethyl acetate and isoamyl acetate exceeded their threshold level in all conditions for the ale strain.

Vicinal diketones

The maximum biosynthetic rates of VDKs were found during the exponential growth phase (Fig. 5). In the case of fermentation at low density, the VDKs reached maximum concentrations when 60% (for the lager yeast) or 50% (for the ale yeast) of the sugar was consumed (end of the exponential phase), and then their concentration decreased, and this for low and high temperature. During the fermentation at high density, the highest level of VDKs was already found when 40% (for the lager yeast) or 25% (for the ale yeast) of the sugar was consumed. The end concentrations of pentanedione and diacetyl decreased with higher fermentation temperature and higher wort density (Table 2). The diacetyl concentration exceeded its flavour threshold at the end of fermentation at low wort density for the lager strain. The fact that the diacetyl concentration is below the threshold value in the fermentation at high wort density, is probably due to the fact that more yeast cells remained in suspension at the end of the fermentation, compared to the other fermentations (Fig. 1e). As diacetyl is a negative flavour compound in lager beers, reduction of diacetyl levels is an important issue in the production of lager beers. For the ale strain, diacetyl concentration at the end of fermentation was always under the threshold level.

Gene expression level

The expression of ten genes involved in the synthesis of flavour-active compounds was quantified in all fermentations. After the cells were harvested and RNA extracted, the expression levels were determined using qPCR as described in the "Materials and methods" section. Gene expression was normalised against the expression level of the *RDN18* (18S) gene. The genes selected for this study are summarised together with their function in Table 3. They are involved in the synthesis of ethanol (*ADH1*), the uptake of leucine, isoleucine and valine (*BAP2*), the synthesis of higher alcohols (*BAT1* and *BAT2*), leucine biosynthesis (*ILV5*), the synthesis (*ATF1* and *ATF2*) and hydrolysis (*IAH1*) of acetate esters and the synthesis of ethyl esters (*EEB1* and *EHT1*) in *S. cerevisiae*.

All genes studied showed different expression profiles during the course of the fermentations depending on temperature and initial wort density (results not shown). *ADH1* was the most highly expressed gene, followed by *EEB1*, *BAP2* and *BAT1* (Table 4). Because *ADH1* is involved in the synthesis of ethanol from pyruvate during alcoholic fermentation, it was expected to be highly expressed under beer fermentation conditions. The lowest expressed genes were *ATF2*, *BAT2* and *EHT1*.

The maximum expression level of the genes *ATF1*, *ATF2*, *IAH1*, *EHT1* and *EEB1* of which the products are involved in the biosynthesis or hydrolysis of esters, was quantified. The expression of *ATF1* and *ATF2* was higher when a higher

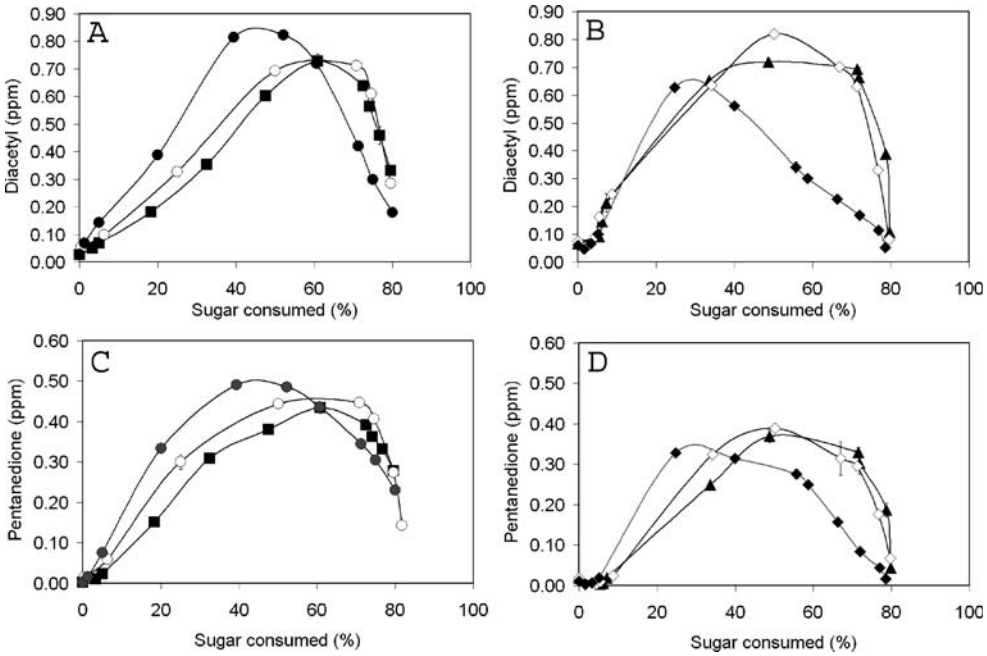

Fig. 5 Vicinal diketones produced in tall tube fermentations with a lager (**a** and **c**) and an ale (**b** and **d**) brewing strain. Production of diacetyl (**a** and **b**) and pentanedione (**c** and **d**). Results are the averages of two independent fermentations. *filled squares*: 12°P 12°C, *empty circles*: 12°P 15°C, *filled circles*: 16°P 15°C, *filled triangles*: 14°P 20°C, *empty diamonds*: 14°P 24°C and *filled diamonds*: 18°P 24°C

Table 3 Genes involved in aroma biosynthetic pathways whose expression was analyzed in this work

Gene	Name	Metabolic pathway
RDN18	18S ribosomal RNA	Component of the 40S ribosomal subunit
ADH1	Alcohol dehydrogenase	Production of carboxylate esters in fermentation
BAP2	Branched-chain amino acid (aa) permease	Uptake of leucine, isoleucine and valine
BAT1	Mitochondrial branched aa aminotransferase	Aa catabolism and branched aa biosynthesis
BAT2	Cytosolic branched aa aminotransferase	Aa catabolism and branched aa biosynthesis
ILV5	Acetohydroxyacid reductoisomerase	Leucine biosynthesis
ATF1	Alcohol acetyltransferase	Acetate ester production
ATF2	Alcohol acetyltransferase	Acetate ester production
IAH1	Isoamyl acetate hydrolase	Isoamyl acetate hydrolyzing esterase
EEB1	Acyl-coenzymeA:ethanol *O*-acyltransferase	Ethyl ester production
EHT1	Ethanol hexanoyl transferase	Ethyl ester production

temperature or a higher initial density was used (Table 4 A). To determine if the maximum expression level of *ATF1* and *ATF2* correlated with the end concentration of the acetate esters, the Pearson product moment correlation coefficient was calculated (Table 5A). A Pearson correlation coefficient between 0.5 and 1 indicates a significant correlation between two variables. The maximum expression level of *ATF1* and *ATF2* clearly correlated with the end concentration of ethyl acetate, isoamyl acetate and phenyl ethyl acetate. The correlation was even stronger for *ATF2* expression, compared to *ATF1* expression. This means that the higher the *ATF1* and *ATF2* expression, the higher the production of acetate esters. As a higher expression of *ATF1* and *ATF2* is correlated with a higher acetate ester production, the *ATF1* and *ATF2* expression level can be used as a tool to predict total acetate ester production.

There was no correlation between the maximum *IAH1* expression level and the end concentration of ethyl acetate, isoamyl acetate and phenyl ethyl acetate (results not shown).

As the maximum *EEB1* expression level was much higher than the maximum *EHT1* expression under fermentation conditions, *EEB1* would appear to be the most important gene for ethyl ester biosynthesis (Table 4A). The maximum expression level of *EEB1* was correlated with the end concentration of ethyl hexanoate (r=60), but there was no correlation with the end concentration of ethyl octanoate and decanoate (Table 5B). So changes in the expression level of *EEB1* are apparently not the cause of changes in the concentrations of longer ethyl esters. This lack of correlation indicates that *EEB1* expression level is not the primary factor for ethyl ester production, as already concluded previously (Saerens et al. 2006, 2008). Precursor availability appears to play a more important role in ethyl ester production than the expression level of *EEB1*. For *EHT1* expression levels, there seems to be a strong negative correlation with the end concentrations of ethyl octanoate and decanoate. As Eht1 has both synthesis and hydrolysis activity towards ethyl esters (Saerens et al. 2008), this appears to indicate that in vivo the esterase activity is the main factor determining ethyl octanoate and decanoate production.

The maximum transcript levels of *BAP2*, *BAT1*, *BAT2* and *ADH1* were also quantified (Table 4B). *BAP2* maximum expression levels were high, compared to *BAT1* and *BAT2*, indicating that uptake of amino acids is an important process during fermentation. *BAT1* maximum expression levels were significantly higher than *BAT2* maximum expression levels and increased when a higher temperature or wort density was applied. The end concentration of all higher alcohols could be correlated to the expression level of *BAT1*, but not to the expression levels of *BAT2* or *BAP2*. The correlation between the maximum expression level of *BAT1* and the end concentration of the higher alcohols was as strong as the correlation of the maximum expression level of *ATF1* and *ATF2* with the acetate ester levels. The strongest correlation of the expression of *BAT1* was with propanol levels.

The expression of *ILV5* could not be linked to diacetyl production. One would expect that a higher level of *ILV5* expression might decrease the formation of diacetyl since less α-acetolactate would be available for escape from the cell. However, under our conditions, we could not observe such an effect.

Discussion

The purpose of this study was to determine the influence of wort density and fermentation temperature on the production of major yeast-derived aroma compounds and on the expression level of ten genes involved in aroma compound production.

Effect of wort gravity on aroma compound formation

Our results show that higher initial wort gravity enhances, in particular, formation of acetate esters. At the high density (16°P and 18°P), acetate ester and also ethyl hexanoate concentrations were significantly higher than at the low density (12°P and 14°P). When a higher wort density is used, ethyl acetate, isoamyl acetate and ethyl hexanoate exceed their threshold level in the

Table 4 Maximum expression levels of *ATF1*, *ATF2*, *EEB1*, *EHT1*, and *IAH1* and B) *ADH1*, *BAT1*, *BAT2*, *BAP2*, and *ILV5*, determined by qPCR analysis in fermentations performed with the lager yeast (12°P 12°C, 12°P 15°C and 16°P 15°C) and the ale yeast (14°P 20°C, 14°P 24°C or 18°P 24°C)

Parameter	Group A					Group B				
	ATF1	ATF2	EEB1	EHT1	IAH1	ADH1	BAT1	BAT2	BAP2	ILV5
12°P 12°C	0.017±0.001	0.0020±0.0003	0.091±0.015	0.0012±0.0001	0.013±0.001	0.063±0.018	0.011±0.001	0.004±0.000	0.069±0.004	0.071±0.005
12°P 15°C	0.041±0.005	0.0030±0.0007	0.096±0.013	0.0017±0.0010	0.022±0.005	0.097±0.011	0.011±0.001	0.005±0.001	0.071±0.006	0.073±0.004
16°P 15°C	0.075±0.005	0.0039±0.0010	0.108±0.013	0.0006±0.0002	0.032±0.011	0.119±0.031	0.020±0.005	0.005±0.000	0.078±0.006	0.075±0.003
14°P 20°C	0.080±0.000	0.0052±0.0003	0.054±0.005	0.0059±0.0001	0.029±0.001	0.076±0.002	0.025±0.000	0.001±0.000	0.034±0.002	0.006±0.001
14°P 24°C	0.110±0.007	0.0081±0.0025	0.129±0.003	0.0139±0.0003	0.019±0.005	0.081±0.009	0.029±0.006	0.002±0.000	0.080±0.003	0.002±0.000
18°P 24°C	0.130±0.045	0.0094±0.0033	0.141±0.036	0.0132±0.0006	0.057±0.003	0.098±0.001	0.037±0.002	0.005±0.000	0.098±0.001	0.005±0.001

fermentations with the lager and ale strain, and are thus important contributors to flavour. In this way, wort density may likely affect the flavour perception of lager and ale beer. For the ale strain, the concentration of higher alcohols also increased when a higher wort density was used. As isoamyl alcohol and phenyl ethanol exceed their threshold level in all conditions, these higher alcohols also contribute to the flavour of ale beers. One possible mechanism for the effect on acetate ester formation involves the link between higher alcohols and the production of acetate esters. However, as the production of higher alcohols increased with a higher density only for the ale strain, an increase in the level of higher alcohols with higher initial wort density only partially explains the higher flavour ester production measured under this condition.

Effect of temperature on aroma compound formation

For the lager strain, the concentration of most of the quantified aroma compounds increased with higher fermentation temperature, except for ethyl acetate and the VDKs, and this was still true when the values were recalculated to 5.1% (*v*/*v*) ethanol. In particular, the higher alcohols and ethyl esters showed an increase with higher temperature, which fits with earlier studies (Dufour et al. 2003; Landaud et al. 2001; Saerens et al. 2008). For the ale strain, only phenyl ethyl acetate concentration increased when a higher temperature was used. It is possible that the temperature increase was too small for the ale strain to measure clear differences in flavour compounds. However, in industrial conditions, temperatures between 20°C and 24°C are commonly used in fermentations for the production of ale beers.

Aroma-active compounds are volatile, and a certain amount will therefore inevitably evaporate from the fermenting medium. This will be more intense at higher temperatures. Loss by evaporation affects to a higher degree compounds with a lower boiling temperature, such as higher alcohols and ethyl acetate. Compounds with a higher boiling temperature, such as the ethyl esters and 2-phenyl ethyl acetate, will be less affected. However, we observed the largest effect of temperature for phenyl ethyl acetate. Therefore, the differences observed cannot be (solely) explained by the effect of temperature on evaporation and are apparently at least to some extent the result of a direct effect on specific yeast biosynthetic pathways. As only isoamyl acetate exceeded its threshold level at the highest temperature for the lager strain and no significant concentration differences were observed for the ale strain, the effect of fermentation temperature on the perceived aroma profile is likely to be limited.

Gene expression level and aroma compound formation

The maximum expression levels of ten flavour biosynthesis genes were analysed to find a correlation between gene ex-

Table 5 Correlation between gene expression and flavour compound production

Parameter	Ethyl acetate	Isoamyl acetate	Phenyl ethyl acetate	Ethyl hexanoate	Ethyl octanoate	Ethyl decanoate	Propanol	Isobutanol	Isoamyl alcohol	Phenyl ethanol
Group A										
ATF1	0.92	0.88	0.91							
ATF2	0.96	0.92	0.97							
Group B										
EEB1				0.60	−0.15	−0.24				
EHT1				0.46	−0.80	−0.88				
Group C										
BAT1							0.97	0.91	0.89	0.90

Calculation of the Pearson product moment correlation coefficient (r) to define the extent of a linear relationship between the maximum gene expression level and the final concentration of a flavour compound in all six conditions tested

pression and flavour compound concentration. The maximum expression levels of *ATF1* and *ATF2* were clearly correlated with the end concentration of acetate esters. This means that a higher production of acetate esters can be explained by a higher *ATF1* and *ATF2* expression. The *ATF1* and *ATF2* expression level can thus be used as a tool to predict total acetate ester production. These findings fit with earlier results, obtained by Verstrepen et al. (2003c), which showed that *ATF* transcription is a limiting factor for acetate ester synthesis. The fact that *ATF1* is induced by glucose and nitrogen compounds [as a target of the Ras/cAMP/PKA and the fermentable growth medium-induced (FGM) pathway; Thevelein and de Winde 1999; Verstrepen et al. 2003a] explains the increase in *ATF1* expression at higher wort density and the resulting increase in acetate ester concentration. In contrast, Molina et al. (2007) found no correlation between expression of *ATF1* and *ATF2* and the production of any ester compound during wine fermentation. They concluded that under wine fermentation conditions, ester production was largely regulated at the posttranscriptional level.

The maximum expression level of *EEB1* was only correlated with the end concentration of ethyl hexanoate, but not with the end concentration of the other ethyl esters. Therefore, changes in ethyl ester levels cannot be explained by differences in the expression level of *EEB1*. Recent work in our laboratory showed that the fatty acid precursor level is likely the major limiting factor for ethyl ester production rather than the activity of the biosynthetic enzymes (Saerens et al. 2008). The results in the present study are in agreement with this idea. However, there seems to be a strong negative correlation between *EHT1* expression levels and the concentration of ethyl octanoate and decanoate. As Eht1 has synthesis and hydrolysis activity towards ethyl esters (Saerens et al. 2008), lower esterase activity could be the cause of higher ethyl octanoate and decanoate production. In vitro studies showed that purified Eht1 had an optimal esterase activity with *p*-nitrophenyl hexanoate and then decreased gradually for longer *p*-nitrophenyl esters (Saerens et al. 2006). On the other hand, in our fermentations, Eht1 seems to have a preference for esterase activity towards longer ethyl esters (ethyl octanoate and decanoate), in vivo.

The expression level of *BAT1* showed a significant correlation with the production of higher alcohols. According to Yoshimoto et al. (2002), isoamyl alcohol production correlates with the expression of *BAT1*. Lilly et al. (2006) showed that modification of *BAT1* gene expression in wine yeast had an impact on the production of all higher alcohols, but especially on isoamyl alcohol and isobutanol. The higher the expression of the *BAT1* gene, the higher the production rate of both higher alcohols. Both temperature and a higher initial wort density increase the expression of *BAT1* and also the production of higher alcohols. An increase in initial wort density enhances the FAN level and expression of *BAT1* increases with increasing nitrogen level. This explains the increased expression of *BAT1* we observed in the fermentation with higher initial wort density.

In conclusion, different volatile aroma profiles were produced during the fermentations with different wort densities. A higher wort density increases acetate ester production, associated with sweet fruity notes for both a lager and an ale brewing yeast strain. For the ale strain, a higher wort density also increased higher alcohol concentration, associated with floral, sweetish aromas. Our results clearly demonstrate that the initial wort density can affect the final concentration of important volatile aroma compounds, thereby contributing to a different flavour perception for both lager and ale beers. Hence, by altering wort density, beneficial adaptation of the flavour of beer may be possible. The results in this study also indicate that this effect appears to be due at least in part to differential expression levels of specific genes involved in the biosynthesis of aroma compounds. This suggests that analysis of gene expression level may help to predict the effect of fermentation parameters on flavour profile and thus can help brewers to identify yeast strains that would produce the desired amounts of esters and higher alcohols in accordance with specific consumer preferences.

Acknowledgements We thank Lynn Stichelbout for excellent help with the qPCR experiments. This work was supported by a grant from the Institute for the Promotion of Innovation by Science and Technology in Flanders (IWT Flanders; SBO project IWT050148) to J.M. Thevelein and F.R. Delvaux.

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