

Characteristics of High Cell Density Fermentations with Different Lager Yeast Strains

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ABSTRACT

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To improve the productivity of the beer fermentation process, several strategies can be adopted. One of these promising strategies could be the increase of suspended yeast cells in the reactor. Therefore, the fermentation characteristics of 11 lager yeast strains were studied in normal pitched worts (20×10^6 cells/mL) (LD) and in worts with a four-fold higher pitching rate (HD). The fermentation rate was 2-4 times increased when high initial cell levels were used. The net yeast growth was somewhat similar between the LD and the HD fermentations, although the FAN uptake level was about 35% higher in the HD fermentations compared with LD. High viabilities were observed throughout the fermentations with high cell loadings. HD fermentations resulted in higher concentrations of all the measured fusel alcohols and higher maxima and residual concentrations of total diacetyl were observed. In contrast, higher levels of most of the esters were found at the normal pitching rate, although the results of isoamyl acetate were not significant. With the help of "Principal Component Analysis", it became clear that the cell density had an important influence on the flavour profile, but that yeast specific preferences could not be overlooked as they determined the sensitivity of the yeast to the application of higher cell densities.

Key words: Fermentation, flavour compounds, high gravity brewing, pitching rate, *Saccharomyces cerevisiae*, yeast metabolism.

INTRODUCTION

In the traditional production of lager beer, the fermentation process takes about 1-2 weeks before entering a maturation period of 1-3 weeks. As a consequence, fermentation and maturation are the most time-consuming steps in the production of beer. Therefore, an important objective of modern fermentation science and technology is to produce an acceptable and similar end product with great time savings.

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In the traditional production of lager beer, yeast strains are typically pitched at a rate of 1 to 1.5 million cells/mL wort per degree Plato of original wort gravity¹². Subsequently, the wort is fermented at temperatures between 10-15°C⁶. During the primary fermentation, wort sugars are converted into ethanol and CO₂ and yeast biomass is generated. In addition, a complex mixture of flavour-active secondary metabolites is produced, of which the higher (or fusel) alcohols and esters are the most important.

Isoamyl alcohol (alcoholic, banana; threshold (TH): 70 ppm²⁴) and 2-phenylethanol (rose-like; TH: 40-125 ppm^{14,24}) can be found around their threshold concentrations in lager beer and can thus contribute significantly to the flavour of lagers. Other higher alcohols, such as propanol and isobutanol contribute to the alcoholic perception of the beer. Higher alcohols are synthesised by yeast during fermentation via the catabolic pathway from amino acids (Ehrlich) and the anabolic pathway from sugars (Genevois)⁹. The major esters in beer are ethyl acetate (solvent-like; TH: 30 ppm²⁴), isoamyl acetate (banana; TH: 1.2 ppm²⁴), ethyl caproate (apple, aniseed; TH: 0.21 ppm²⁴), ethyl caprylate (apple; TH: 0.9 ppm²⁴) and phenylethyl acetate (roses, honey; TH: 3.8 ppm²⁴). Esters are desirable components of beer when present in appropriate quantities and contribute to the fruity and flowery character of the beer. They are formed by the condensation reaction between acetyl/acyl-CoA and ethanol/higher alcohols catalysed by the alcohol acyltransferases of yeast²³.

Next to these positive flavour compounds, the vicinal diketones, such as diacetyl (buttery; TH: 0.08-0.15 ppm²⁴) and pentanedione (TH: 0.9 ppm) can cause off-flavours. Pentanedione has a threshold which is approximately ten times higher than that of diacetyl and is thus quantitatively of minor importance. Diacetyl is formed during fermentation by an extracellular oxidative decarboxylation of α -acetolactate, which leaks from the biosynthesis pathway of valine from pyruvate³³. Subsequently, yeast reduces diacetyl to the flavour inactive acetoin and further to 2,3-butanediol³. The reduction can occur quickly when sufficient yeast is present.

Because the complex flavour profile is closely related to amino acid metabolism and thus the growth of the yeast cells, differences in the growth metabolic state between traditional and accelerated fermentation processes are

most probably responsible for the majority of alterations in the beer flavour.

Increasing process parameters such as temperature and agitation results in a significant decrease of the fermentation time, but alteration of these variables also has a pronounced negative effect on the flavour balance of the beer^{5,11,13,17,30}. On the contrary, an increase of the initial cell concentration is often effective in decreasing the fermentation time without major deleterious influences on beer quality^{15,26,28}. Former studies about the effect of pitching rate on beer composition are however often contradictory and some of these differences may be attributed to differences between yeast strains. Edelen et al.¹² showed that the net yeast growth remained constant for higher pitching rates and higher levels of esters and some higher alcohols were observed with lower pitching rates. Erten and co-workers¹⁵ found lower levels of both higher alcohols and vicinal diketones with increasing pitching rates, whereas the ester levels, with the exception of isoamyl acetate, remained constant. Finally, Posada et al.²⁸ did not find any important differences in flavour compound concentrations between normal and high pitching rates.

In this study, fermentations with high and normal pitching rates were carried out with different lager yeast strains to investigate the impact of high cell densities on fermentation performance and beer quality.

MATERIALS AND METHODS

Yeast and wort

All experiments were carried out with industrial lager brewing strains of *Saccharomyces cerevisiae* (*carlsbergensis*) (CMBSPV01-10) (Katholieke Universiteit Leuven, Centre for Malting and Brewing Science, Heverlee, Belgium), maintained on wort agar (LAB M, International Diagnostic Group, Bury, UK) at 4°C. Additionally, early stationary phase cells of yeast strain CMBSPV03 (grown on YPD) were plated at 10⁷ cells on YNB plates (BD Difco) plus 2% glucose with 1 mM 5,5,5-trifluoro-leucine (TFL) and incubated at 30°C for 48 h⁸. Yeast colonies were isolated on a second plate and this TFL-mutant strain was called CMBSPV03TFL.

Sterile all-malt hopped wort (15°P) (69% maltose, 18% maltotriose, 9% glucose, 4% fructose) with a FAN content of 254 ppm was used throughout the study.

Propagation and fermentation conditions

For yeast propagation, single yeast colonies were taken from stock plates and inoculated into 8 mL 12°P all-malt wort (in triplicate). After incubation at 25°C for 24 h with orbital shaking at 180 rpm, the suspensions were transferred to 200 mL of wort (12°P) in 250-mL Erlenmeyer flasks and incubated at 20°C for 48 h on an orbital shaker at 140 rpm. Afterwards, the three suspensions were brought into 3 L wort of 15°P and the culture was statically incubated for 3 days and afterwards put on ice for 24 h. Finally, the wort was decanted and the remaining yeast slurry was counted (Thoma haemocytometer) before the required amount was pitched in the wort.

All fermentations were carried out in duplicate, in EBC tall tubes, containing 1.8 L sterile 15°P wort medium. The

wort was aerated by filling the headspace with sterile air followed by intensive mixing by inverting the tubes twenty times¹. For each yeast strain, two different pitching rates were used: the normal pitching rate (low cell density: LD) of 20 × 10⁶ viable cells/mL and the high pitching rate (high cell density: HD) of 80 × 10⁶ cells/mL. The fermentations were performed at 15°C and were monitored daily for the normal cell density fermentations and more frequently for the high cell density fermentations.

Fermentation analysis

The number of suspended yeast cells was counted by haemocytometry. Viability was assessed using the methylene blue staining method¹. The density of the fermenting medium was measured with a handheld density meter (DMA 35N, Anton Paar, Graz, Austria), but the final extract and alcohol content were measured with the DMA 4500 density analyzer and Alcoyser Plus (Anton Paar, Graz, Austria).

Free Amino Nitrogen (FAN) was determined by a ninhydrin-based method¹⁰.

Volatile compound concentrations were determined by headspace gas chromatography. Collected samples were cooled on ice, and after centrifugation, 5 mL of the filtered, undiluted supernatant was transferred to a vial. The vials were analysed with a calibrated Autosystem XL gas chromatograph with a headspace autosampler (HS40; Perkin Elmer, Norwalk, USA) and equipped with a Chrompack-Wax 52 CB column (length: 50 m; 0.32 mm ID; layer thickness: 1.2 µm; Varian, Palo Alto, CA). 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 kept at 50°C for 5 min, increased to 200°C at 5°C/min and was held at that temperature for 3 min. Detection of dimethyl sulphide (DMS), esters and higher alcohols was established with a flame ionisation detector (FID); total diacetyl (diacetyl + α -acetolactate) was detected with an electron capture detector (ECD). The FID and ECD temperatures were kept constant at 250 and 200°C respectively. Analyses were carried out in duplicate and the results were analysed with Perkin Elmer Turbochrom Navigator software and were recalculated to 5% (v/v) ethanol.

Statistical analysis

Data of flavour compounds were analysed for statistical significance by parametric t-tests for paired samples, using the software XLSTAT 2007.6 for MS excel. "Principal Component Analysis" (PCA) was carried out using the Unscrambler 9.7 software¹⁶.

RESULTS AND DISCUSSION

Fermentation performance

The time periods needed to reach 3.5°P for the different fermentations are depicted in Fig. 1. The extract decrease was accelerated significantly when using a four-fold higher pitching rate. The average fermentation time decrease was about three-fold, but the increase in fermentation rate was very yeast strain dependent. The yeast strains CMBSPV04, CMBSPV06 and CMBSPV10

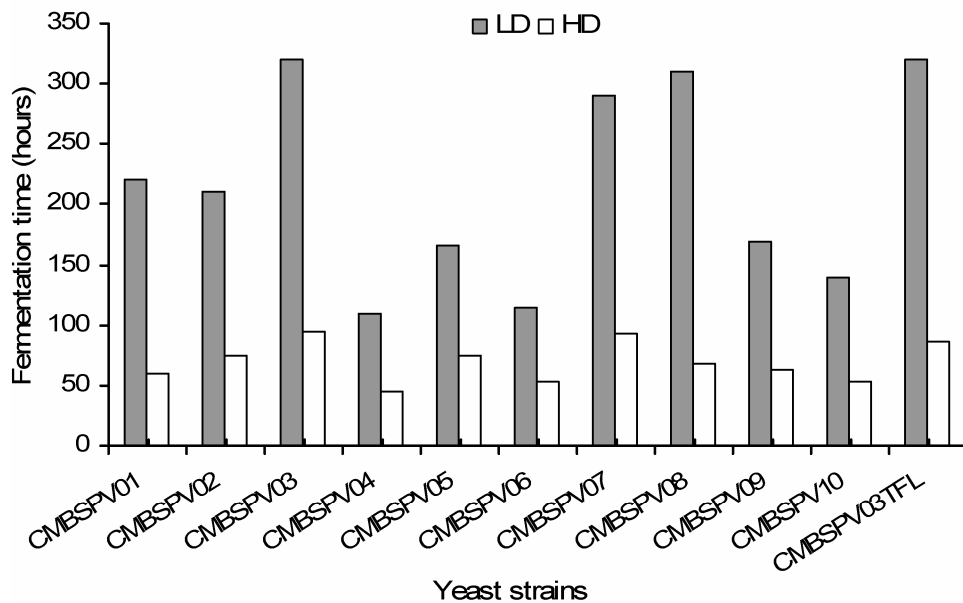


Fig. 1. Duration of the fermentations with 11 yeast strains to reach 3.5°P. LD: low cell density; HD: high cell density.

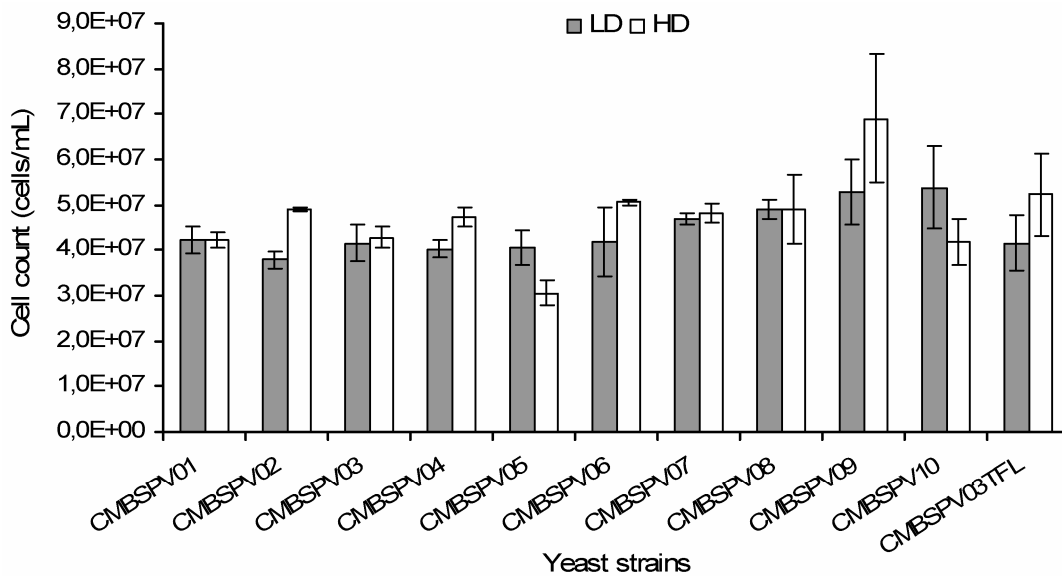


Fig. 2. Net yeast growth (the maximum yeast cell count - initial yeast cell concentration) of the LD and HD fermentations with 11 different yeast strains. The error bars show the standard deviations of the duplicate fermentations.

were the fastest fermenting strains in both types of fermentations. In contrast, CMBSPV03, CMBSPV07, CMBSPV08, and CMBSPV03TFL, were the slowest fermenting yeasts.

All the LD and HD fermentations with different yeast strains followed a normal fermentation curve. The average apparent degree of attenuation (HD: $79.3 \pm 0.7\%$; LD: $78.6 \pm 1.0\%$) and the average ethanol production (HD: $6.52 \pm 0.06\%$; LD: $6.55 \pm 0.15\%$) were not significantly different between the LD and HD fermentations. A slightly lower pH for all the HD fermentations compared with the normal fermentations was observed (HD: 4.21 ± 0.07 ; LD: $4.36 \pm 0.05\%$).

As expected, the maximum yeast cell counts were higher in the case of the higher pitching rate. In Fig. 2, the net growth (the total maximum yeast cell count - initial inoculum size) of the yeast cell populations during normal and high cell density fermentations is illustrated. The graph shows that yeast growth was strain dependent, but for most of the investigated yeasts, the net growth of the LD fermentations was not significantly different from that of the HD fermentations. Hence, the same amount of new yeast cells was generated during the LD and HD fermentations. The yeast HD population at the end of fermentation will then have a larger percentage of 'older' cells. Edelen et al.¹² explained this phenomenon by the

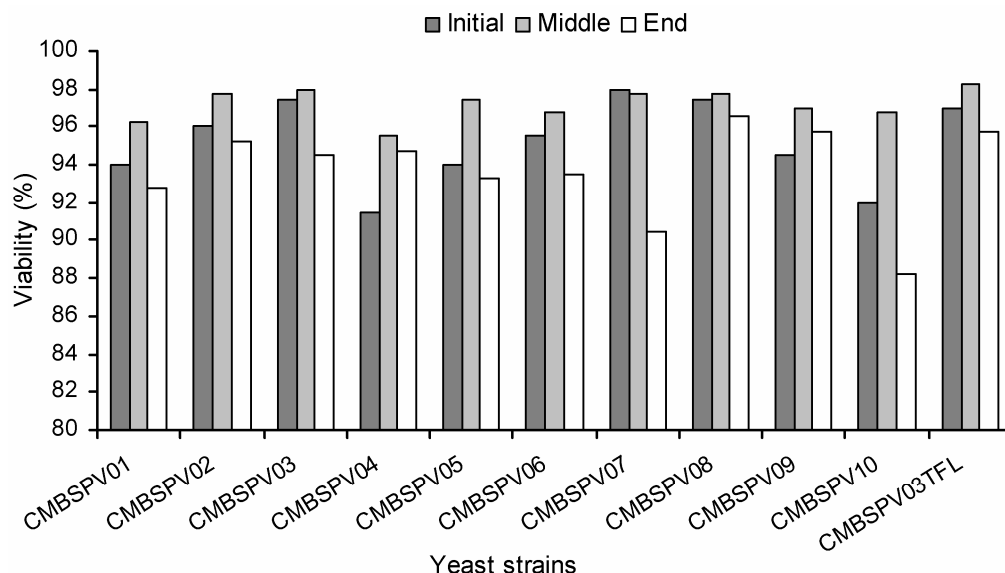


Fig. 3. Viability of the HD fermentations with 11 different yeast strains at the beginning, in the middle and at the end of fermentation.

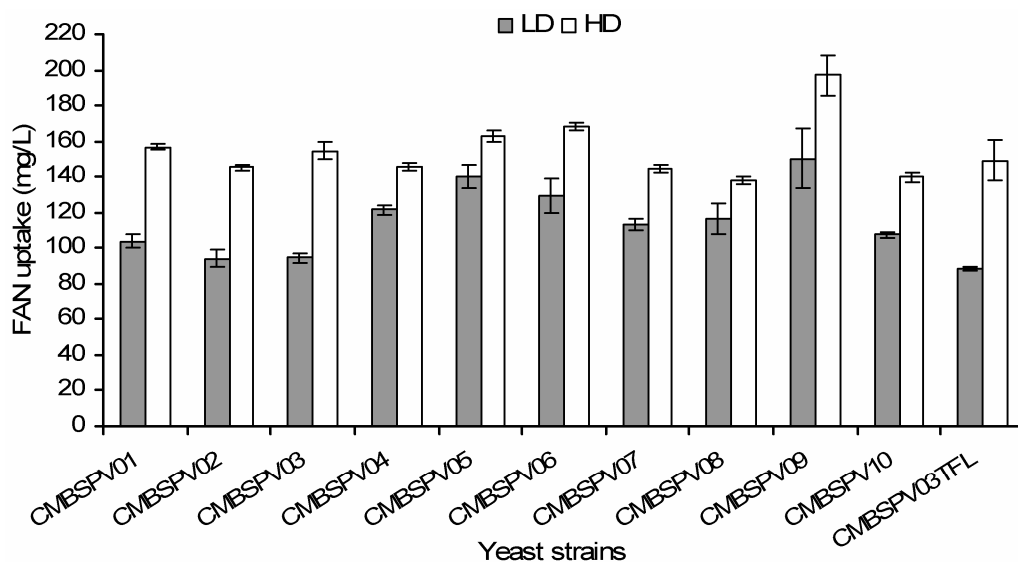


Fig. 4. FAN uptake (initial FAN – final FAN) of LD and HD fermentations with 11 different yeast strains. The error bars show the standard deviations of the duplicate fermentations.

depletion of oxygen as the limiting factor for yeast growth at higher pitching rates.

The percentage of viable cells was monitored at the beginning, in the middle and at the end of the HD fermentations and is illustrated in Fig. 3. The results show that viability stayed above 93%, except for CMBSPV07 (90.5%) and CMBSPV10 (88.3%). The viability in the high cell concentrated fermentations thus remained acceptable.

FAN amounts were determined at the beginning and at the end of fermentation (Fig. 4). For all the investigated yeast strains, FAN consumption was higher when a four-fold higher pitching rate was used, with an average increase of 35%.

Impact of HD on flavour

Flavour profiles of LD and HD fermentations. Some of the most important compounds, characterising the flavour profile of the resulting beers, are presented in Table I. The flavour profile of the beers was clearly yeast strain dependent. On the data of all measured flavour compounds, pairwise compared t-tests were executed to find significant differences between the LD and HD fermentations.

The higher pitching rate resulted in a significant increase of the higher alcohols propanol, isobutanol and phenyl ethanol. At first sight, isoamyl alcohol showed no dependence for cell concentrations during beer fermenta-

Table I. Flavour compound concentrations at the end of the fermentations, recalculated to 5% (v/v) alcohol, from the LD and HD fermentations with different yeast strains.

Flavour compounds (mg/L)	Different fermentations																						Sig. ^b
	CMBSPV01		CMBSPV02		CMBSPV03		CMBSPV04		CMBSPV05		CMBSPV06		CMBSPV07		CMBSPV08		CMBSPV09		CMBSPV10		CMBSPV03TFL		
	LD	HD	LD	HD	LD	HD	LD	HD	LD	HD	LD	HD	LD	HD	LD	HD	LD	HD	LD	HD	LD	HD	
Higher alcohols																							
Propanol	11.5	12.4	11.5	12.6	10.1	12.4	10.8	12.7	16.1	18.2	12.5	13.2	11.5	12.1	13.0	13.0	12.8	16.1	12.0	11.5	9.3	10.8	**
Isobutanol	11.9	15.2	9.8	13.7	7.5	10.3	9.0	11.7	16.7	19.7	9.3	12.5	15.5	20.1	12.5	12.8	8.2	12.4	12.6	13.0	6.5	7.4	**
Isoamyl alcohol	51.2	56.8	50.2	61.0	43.3	53.6	46.3	51.4	67.7	70.0	44.1	47.7	63.4	67.6	49.4	48.2	47.7	57.3	48.2	48.6	106.3	133.2	ns ^c
Phenylethanol	25.0	33.8	21.5	28.8	20.5	23.9	24.2	31.0	34.4	40.6	27.2	29.3	31.3	43.5	19.9	24.8	20.4	34.2	24.0	30.2	24.2	28.6	**
Esters																							
Ethyl acetate	26.2	22.6	24.9	22.0	23.2	22.8	25.3	20.4	31.9	23.9	34.9	25.1	37.5	35.4	38.2	32.9	35.5	26.4	34.7	27.2	24.9	23.6	**
Isoamyl acetate	1.54	1.27	1.68	1.47	0.88	0.98	2.29	1.77	2.52	1.26	2.77	1.97	2.83	2.85	2.73	2.33	2.15	2.4	2.59	2.12	2.66	2.72	ns
Phenylethyl acetate	0.46	0.42	0.43	0.45	0.28	0.36	0.71	0.58	0.80	0.48	1.00	0.85	0.90	1.12	0.73	0.71	0.67	0.96	0.91	0.78	0.39	0.41	ns
Ethyl caproate	0.167	0.146	0.203	0.195	0.191	0.143	0.194	0.186	0.217	0.123	0.245	0.232	0.203	0.169	0.174	0.154	0.175	0.146	0.207	0.178	0.189	0.145	**
Ethyl caprylate	0.749	0.358	0.458	0.285	0.511	0.323	0.282	0.232	0.491	0.258	0.554	0.249	0.620	0.427	0.831	0.600	0.659	0.379	0.605	0.420	0.494	0.321	**
Ethyl caprate	0.126	0.050	0.145	0.062	0.053	0.026	0.055	0.027	0.065	0.041	0.088	0.031	0.076	0.054	0.149	0.083	0.035	0.036	0.139	0.071	0.046	0.031	**
DMS	0.031	0.022	0.019	0.016	0.019	0.022	0.014	0.012	0.027	0.023	0.022	0.020	0.038	0.033	0.068	0.053	0.041	0.025	0.032	0.021	0.021	0.020	*
Max. diacetyl ^a	0.289	0.458	0.508	0.729	0.258	0.351	0.344	0.526	0.365	0.548	0.244	0.396	0.235	0.417	0.246	0.426	0.322	0.401	0.309	0.418	0.804	1.007	**
Diacetyl	0.006	0.063	0.006	0.151	0.028	0.068	0.085	0.336	0.038	0.097	0.026	0.071	0.015	0.076	0.006	0.015	0.010	0.121	0.025	0.095	0.027	0.223	**

^a Maximum diacetyl concentrations are peak values measured during fermentation and are not recalculated to 5% (v/v) alcohol.

^b Significance: * and ** indicate a significant difference between LD and HD with $\alpha = 0.05$ and $\alpha = 0.01$ respectively according to parametric t-tests for paired samples; ns: not significant.

^c When CMBSPV03TFL was excluded from the data set, a significant difference with 99% probability was obtained between LD and HD fermentations.

tion. However, when the mutant yeast strain CMBSPV03-TFL was excluded from the pairwise comparison, a significant difference (with $\alpha = 0.01$) was obtained (see next paragraph).

Significantly less ethyl acetate and ethyl esters were produced during the HD fermentations. On the other hand, no significant differences for phenylethyl acetate and isoamyl acetate were observed.

Noteworthy, significant differences in the DMS concentration between the LD and HD beers were observed, although the same wort was used throughout the study. DMS is considered to be an important compound in lager beers when present at concentrations above 0.03 ppm and has a cabbage-like flavour²⁴. The two main routes leading to the formation of DMS are the thermal degradation of S-methylmethionine into DMS and homoserine during malting and brewing and the reduction of dimethyl sulphoxide (DMSO) by yeast during fermentation². This reduction appears to be very yeast strain dependent. The higher DMS concentrations in the LD beers could be explained by the higher pH during LD fermentation, less removal of DMS by fermentation gases (due to less vigorous fermentation properties), or more efficient conversion from DMSO^{2,22}.

Diacetyl was present in significantly higher amounts at the end of the HD fermentations. This was probably due to higher production of α -acetolactate during fermentation (higher total diacetyl maxima, see Table I) and the shorter fermentation times, resulting in incomplete conversion of α -acetolactate to diacetyl, which is the rate-limiting step in the removal of diacetyl from the fermenting wort. All the LD fermentations were long enough to reduce the diacetyl content below the threshold.

Flavour profile of CMBSPV03TFL. The yeast strain CMBSPV03TFL was performing similarly to its parent strain CMBSPV03 during fermentation (see Figs. 1, 2, 3

and 4) except for the concentration of isoamyl alcohol and consequently isoamyl acetate, which were 2.3-2.8 times higher in comparison with CMBSPV03. This result was already observed in other studies on wine^{4,8} and beer²¹, where it was shown that resistance against 5,5,5-trifluoro-leucine (TFL) is due to dominant mutations on the gene *LEU4*, which encodes for α -isopropylmalate synthase I, an enzyme present in the biosynthetic pathway of leucine from pyruvate²⁵. In these TFL-resistant mutants, the enzyme becomes resistant to feedback inhibition by leucine, resulting in an increased formation of isoamyl alcohol and isoamyl acetate. This mutant strain was included in the selection to observe the effect of amino acid independent formation of the positive flavour compounds isoamyl alcohol and isoamyl acetate during HD and LD fermentations, to counteract the negative impact of higher pitching rates on flavour. Interestingly, not only isoamyl alcohol and isoamyl acetate increased in this mutant: the production of total diacetyl during fermentation was also about 3 times higher in the mutant CMBSPV03TFL, compared to CMBSPV03. This could be explained by altered expressions, caused by the activated transcriptional factor Leu3p, of *ILV2* and *ILV5*, which encode the α -acetolactate synthase and the α -acetolactate reductoisomerase respectively, as a result of the mutation³¹. The activity of these enzymes determines the production of α -acetolactate during fermentation, which is converted to diacetyl by an oxidative decarboxylation³³.

PCA of the acquired flavour compound data. PCA is a descriptive statistical technique, which allows the visualisation of the main variability of the data set¹⁸. The main goal of applying PCA to the data was to get an overall view of the differences between the LD and the HD fermentations on the one hand and the yeast strain dependent variations on the other hand. After scaling and weighting of the data and excluding CMBSPV03TFL,

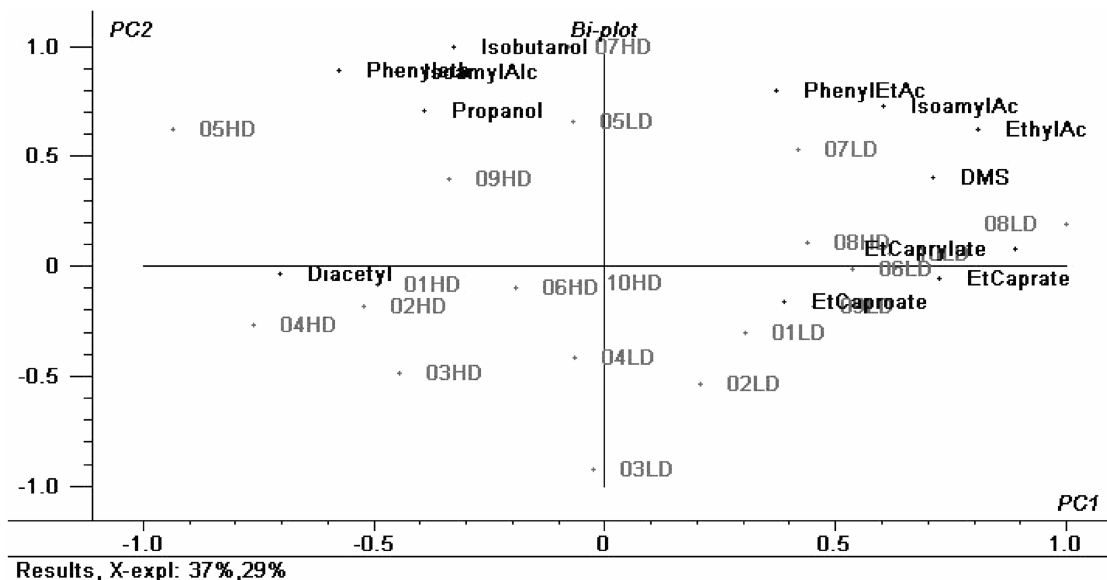


Fig. 5. PCA plot for flavour compound data of LD and HD fermentations with 10 different yeast strains (CMBSPV03TFL was excluded from the data set).

because of its outlying characteristics, a PCA plot was generated. In Fig. 5, PC1 and PC2 are presented, which account for 66% of the information.

It appears that PC1 described the effect of pitching rate as the LD fermentations were depicted on the right of the plot and a translation to the left was observed for all the yeast strains when HD fermentations were performed. As already described earlier, the concentrations of the esters and DMS seemed to correlate positively with LD, while the opposite was true for diacetyl and the higher alcohols. However, yeast specific characteristics should not be overlooked as the HD beer of CMBSPV08 had some clear preferences of LD beers made with other yeast strains as depicted in the plot. The LD beers of CMBSPV04 and CMBSPV05 on the other hand showed similarity with other HD beers. In addition, the extent of the translation along PC1 was yeast strain dependent, which indicates that the extent of the effect of applying HD fermentations will depend on the yeast strain applied. The distance between two points in the PCA plot indeed gives an idea of the similarity of the beers.

PC2 gave an important part of the information as it still accounts for 29% of the information included in the original data set. This PC did not correlate with the applied cell density, but gave a measure of the difference between the flavour compound formation by the yeasts. For example CMBSPV05 and CMBSPV07 produced more acetate esters and alcohols independently of the pitching rate compared to the other yeasts.

CONCLUSIONS

The response of the use of high cell concentrations on fermentation performance proves to be very yeast strain dependent. Therefore, the use of higher pitching rates could offer a solution to accelerate the fermentation of wort to beer significantly without serious alterations in flavour, on condition of selecting the appropriate yeast strain.

High pitching rates result in lower relative yeast growth. This implicates that the yeast population at the end of fermentation is relatively older than the yeast population of normal pitched wort. This could have important economical and technological consequences for the physiology of yeast during subsequent fermentations with the same yeast population²⁹. Therefore, it would be interesting to study the influence of pitching rate on yeast physiology in more detail.

One main problem of most of the accelerated fermentation systems remains diacetyl, because of the higher production of its precursor α -acetolactate and the incomplete chemical conversion of α -acetolactate to diacetyl, causing incomplete removal of diacetyl by yeast, due to the short residence times⁷. However, several strategies can be followed to decrease the diacetyl content in beer, such as accelerated maturation using immobilised yeast²⁷, supplementation of α -acetolactate decarboxylase to the wort¹⁹ or the use of genetically manipulated yeast strains^{20,32}. Next to this, the implementation of TFL-resistant mutants, which produce higher amounts of isoamyl alcohol and isoamyl acetate, in HD fermentations, seems pointless, because of the higher production rate of α -acetolactate during fermentation, resulting in a higher diacetyl content at the end of fermentation.

It appears that the pitching rate has an important influence on the flavour profile, but that yeast specific preferences cannot be overlooked as they determine the sensitivity of the yeast to the application of different cell densities. Moreover, the flavour profile still remains mainly determined by the applied yeast strain.

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