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Impact of pitching rate on yeast fermentation performance and beer flavour

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Abstract The volumetric productivity of the beer fermentation process can be increased by using a higher pitching rate (i.e. higher inoculum size). However, the impact of the pitching rate on crucial fermentation and beer quality parameters has never been assessed systematically. In this study, five pitching rates were applied to lab-scale fermentations to investigate its impact on the yeast physiology and beer quality. The fermentation rate increased significantly and the net yeast growth was lowered with increasing pitching rate, without affecting significantly the viability and the vitality of the yeast population. The build-up of unsaturated fatty acids in the initial phase of the fermentation was repressed when higher yeast concentrations were pitched. The expression levels of the genes *HSP104* and *HSP12* and the concentration of trehalose were higher with increased pitching rates, suggesting a moderate exposure to stress in case of higher cell concentrations. The influence of pitching rate on aroma compound production was rather limited, with the exception of total diacetyl levels, which strongly increased with the pitching rate. These results demonstrate that most aspects of the yeast physiology and flavour balance are not significantly or negatively affected when the pitching rate is changed. However, further research is needed to fully optimise the conditions for brewing beer with high cell density populations.

Keywords Fermentation · Brewer's yeast · Yeast metabolism · Yeast physiology · Stress response · Flavour

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Introduction

In the traditional production of lager beer, the fermentation process is the most time-consuming step, which takes about 1–2 weeks before entering the maturation period. Therefore, an important objective of modern fermentation science and technology is to reduce the fermentation time while producing an end product of similar quality and thus allowing great time and money savings. To improve the volumetric productivity of the beer fermentation process, several strategies can be adopted. For example, a major approach was the application of continuous fermentation with immobilised yeast, allowing a large increase in cell density, which resulted in faster fermentation rates. The interest in using immobilised yeast for primary beer fermentation seems to have dropped because of persistent engineering problems, unbalanced beer flavour and unrealised cost advantages (Brányik et al. 2005). However, the main aim of the technology was to improve the productivity by maximising the cell concentration in the reactor. Therefore, another promising strategy may be to enhance the amount of suspended yeast cells in a batch fermentor (i.e. 'the pitching rate'; Okabe et al. 1992; Verbelen et al. 2008). However, an increase in the pitching rate could also have deleterious side effects on the physiological condition

of the yeast population and/or the flavour profile of the beer. The impact of the pitching rate on crucial yeast, fermentation and beer quality parameters has never been assessed systematically.

Yeast physiology and activity before pitching and during fermentation are instrumental in achieving consistent fermentations that result in beers of acceptable quality. During fermentation of wort to beer, several physiological changes occur in the yeast population. In the beginning of fermentation, build-up of unsaturated fatty acids and sterols is essential for a normal growth pattern of the yeast population during the next stage of the fermentation and thus also for a proper overall fermentation process (David and Kirsop 1973). The energy needed for the formation of these essential lipids originates from the breakdown of glycogen, the most important reserve carbohydrate in yeast. Therefore, glycogen levels are a crucial factor for yeast fermentation potency (Quain and Tubb 1982). After the disappearance of oxygen from the wort, glycogen accumulates in the yeast during the exponential growth phase. Maximum glycogen levels are reached at the end of primary fermentation (Boulton 2000). At that moment, growth and cell division are arrested due to dilution of essential lipids over mother and daughter cells (Aries and Kirsop 1977). When the yeast cells stop growing, they go into a quiescent stationary phase. Glycogen provides energy for the cellular maintenance functions during the stationary phase and during the storage phase between cropping and the next pitching (Boulton 2000). Regulation of glycogen content is complex and occurs in part by the yeast cyclic adenosine monophosphate (cAMP)–protein kinase A (PKA) pathway (Smith et al. 1998). The high PKA activity represses glycogen accumulation, but when an essential nutrient is progressively consumed from the growth medium, PKA activity is reduced and glycogen content increases (François and Parrou 2001).

Brewing yeast encounters different kinds of stress during fermentation and yeast handling (Gibson et al. 2007). Exposure to moderate stress may result in the modification of the genetic, metabolic and physiological responses of brewing yeast. In turn, such shifts in cellular function are likely to cause modifications in subsequent fermentation performance. When the stress is severe or the damage has accumulated to intolerable levels, viability starts to drop (Jenkins and Kennedy 2003). Yeast cells can adapt to adverse conditions by several stress response mechanisms (Siderius and Mager 2003). Two major stress response pathways in *Saccharomyces cerevisiae* are the general stress response and the heat shock response. The latter is activated specifically by heat stress. The general stress response can be triggered by different external stress agents, such as exposure to heat, exposure to ethanol, increase in osmolarity, exposure to reactive oxygen species or starvation for essential nutrients. The general stress response is mediated by the transcriptional activators Msn2p and Msn4p, which upon stress exposure become competent to activate transcription of stress responsive element (STRE)-driven genes (Martinez-Pastor et al. 1996; Smith et al. 1998). STRE sequences have been identified in promoter sections of many stress-induced genes, such as heat shock protein (HSP) genes (e.g. *HSP12* and *HSP104*) and genes that contribute to the synthesis (*TPS1*, *TPS2*, *TPS3* and *TSL1*) and degradation (*NTH1*, *NTH2*) of trehalose (Winderickx et al. 1996; Zähringer et al. 1997). In addition, STRE-containing genes contain other regulatory sequences involved in stress-induced transcription, e.g. heat shock elements (HSE) or the antioxidant response element (ARE; Estruch 2000). The stress-induced expression, conferred by STRE, is negatively regulated by the cAMP–PKA pathway (Thevelein and de Winde 1999; Verstrepen et al. 2004). Because accumulation of the disaccharide trehalose is induced in response to a variety of stress conditions, like heat shock, high osmolarity, ethanol and exposure to toxic chemicals, trehalose accumulation can be used as a general indicator for stress on the yeast cells (Majara et al. 1996; Hounsa et al. 1998). At the beginning of fermentation, high glucose levels fully activate the cAMP/PKA pathway, which results in post-translational activation of neutral trehalase and in induction of its *NTH1* gene, and also in repression of the trehalose synthase genes, resulting in reduced levels of trehalose (Zähringer et al. 2000). When glucose is consumed, maltose starts to be assimilated and the PKA activity decreases, resulting in the formation of trehalose (Verstrepen et al. 2004). Under stress conditions, the higher levels of trehalose protect the cells by binding to membranes and proteins (François and Parrou 2001). However, during recovery from stress, trehalose must be hydrolysed rapidly by trehalase, liberating the cellular structures from bound trehalose (Zähringer et al. 2000). The rapid degradation of trehalose may be essential for the resumption of normal cellular activity (Gibson et al. 2007).

The most important organoleptic characteristics in lager beer are the flavour compounds formed by yeast during fermentation of which the higher alcohols, the ‘fruity’ esters (contributing to alcoholic and fruity flavours) and the vicinal diketones and sulphur compounds (which can cause off-flavours) are the most important. Because the metabolism of these flavour compounds is closely related to the growth and physiological state of the yeast, factors that affect yeast metabolism and physiology can easily change beer flavour. Altering process parameters, such as wort composition, temperature, agitation and pitching rate can thus result in significant changes in the beer flavour balance (Okabe et al. 1992; Boswell et al. 2002; Dufour et al. 2003; Saerens et al. 2008).

In a previous study, high cell density fermentations were performed with different lager yeast strains (Verbelen et al. 2008). It was concluded that at a high pitching rate, the fermentation rate was two to four times increased, depending on the yeast strain. High viabilities were observed throughout the different fermentations with high cell loading. The cell density had an important influence on the resulting flavour profile and on specific yeast preferences. In this study, an industrial yeast strain selected from the previous study (Verbelen et al. 2008) was used to investigate systematically the impact of higher inoculum size on yeast fermentation performance. We found that the pitching rate did not negatively influence the physiological condition of the yeast. Only the flavour profile was affected to a certain extent, in particular the total diacetyl concentration. This knowledge will be crucial for further optimisation of the promising technique of high cell density beer fermentation.

Materials and methods

Yeast strain and medium

The experiment was carried out with an industrial lager brewing strain of *S. cerevisiae* (*pastorianus*) (CMBSPV09) (Centre for Malting and Brewing Science, Katholieke Universiteit Leuven, Heverlee, Belgium), obtained from a brewery yeast storage vessel.

Sterile all-malt hopped wort with an extract content of 15 °Plato (°P) (°P: g extract/100 g wort; 68% maltose, 19% maltotriose, 9% glucose, 4% fructose) and with a free amino nitrogen (FAN) content of 404 ppm was made in a pilot brewery and used throughout the study.

Fermentation conditions and sampling

The concentration and viability of the yeast slurry were determined by flow cytometry (YeastCyte, *Bio*DETECT AS, Oslo, Norway) before the required amount was pitched in the wort. Five different pitching rates were applied: (1) 10×10^6 viable cells/ml, (2) 20×10^6 viable cells/ml, (3) 40×10^6 viable cells/ml, (4) 80×10^6 viable cells/ml and (5) 120×10^6 viable cells/ml. All fermentations were carried out in duplicate, in tall tubes (75 cm high, 8 cm internal diameter), 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 20 times. The fermentations were performed at 15 °C and were monitored frequently by withdrawing samples through a narrow sampling tube (15 cm from the bottom) with the aid of N_2 overpressure. Samples were cooled directly on ice and the yeast and fermenting wort were separated by centrifugation (2,800 rpm, 3 min, 2°C). Fermentations were stopped at around 80% apparent degree of fermentation (ADF) and the tubes were cooled down at 2°C for 24 h to sediment the yeast. The supernatant of the tube (beer) was collected and the remaining slurry was resuspended in 1-l cold sterile water, after which samples were taken to characterise the cropped yeast.

Fermentation analysis

Before centrifugation, the number and the viability of suspended yeast cells were counted by flow cytometry (YeastCyte, *Bio*DETECT AS, Oslo, Norway). Dead cells were stained in-line with 10% *v*/*v* propidium iodide in PBS (70355, Sigma).

The specific gravity 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 analyser and Alcolyser Plus (Anton Paar, Graz, Austria).

Free amino nitrogen was determined by a ninhydrin-based method, according to the standard method as defined by the European Brewery Convention (EBC 1998).

The glycerol content of the final beers was determined with a glycerol kit (K-GCROL, Megazyme International, Ireland), according to the manufacturer's instructions.

The bitter compounds were extracted with isooctane from the acidified beers and the absorbance was measured at 275 nm in a quartz cuvette (EBC 1998).

Flavour compound analysis

Volatile compound concentrations were determined by headspace gas chromatography. Five millilitres of the cooled, filtered, undiluted supernatant was transferred to a vial. The vials were heat-treated during 60 min at 60°C to convert all α-acetolactate to diacetyl. The vials were analysed with a calibrated Autosystem XL gas chromatograph with a headspace autosampler (HS40; Perkin Elmer, Wellesley, MA, USA) and equipped with a Chrompack-Wax 52 CB column (length 50 m; 0.32 mm internal diameter; layer thickness 1.2 µm; Varian, Palo Alto, CA, USA). Samples were heated for 16 min at 60°C in the headspace autosampler before injection (needle temperature 70°C). Helium was used as the carrier gas. The oven temperature was kept at 50°C for 7.5 min, increased to 110°C at 25°C/min and was held at that temperature for 3.5 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°C 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.

Yeast physiology

Glycogen and trehalose

The quantification of glycogen and trehalose was based on the method of Neves et al. (1991). Yeast samples were washed three times with cold distilled water. Afterwards, the yeast suspension was filtered over a membrane filter (pore size 0.45 µm) and 25–50 mg (wet weight) of cell pellet was weighed and immediately stored at −80°C. After thawing, the pellets were resuspended in 1 ml of 0.25 M Na_2CO_3 and incubated for 20 min at 95°C. After that, the extractions were cooled down and centrifuged (1 min at 13,000 rpm) and 10 µl of the supernatant was withdrawn and adjusted to pH 5.5 with 5 µl of 1 N acetic acid for trehalose determination. The rest of the extract was resuspended and re-incubated at 95°C for 40 min. After cooling down, 10 µl of the suspension was adjusted with 5 µl of 1 N acetic acid and used for glycogen determination. Trehalose (0, 1, 2, 4 and 8 mM in 0.25 M Na_2CO_3) and glycogen standards (0, 1, 2 and 3 mg/ml in 0.25 M Na_2CO_3) were treated in the same way as the yeast samples, with exception of the extraction.

Trehalose was broken down enzymatically with 25 µl of a trehalase buffer mix, containing 5 µl buffer (300 mM NaAc, 30 mM $CaCl_2$, pH 5.5) and 20 µl *Humicola* trehalase preparation (ca 350 U/ml; kindly provided by the Laboratory of Molecular Cell Biology, Katholieke Universiteit Leuven, Heverlee, Belgium). Glycogen was broken down with 25 µl of an α-amyloglucosidase buffer mix, containing 1.2 mg α-amyloglucosidase (500 U, Roche Diagnostics, Indianapolis, USA) per 100 ml of buffer (80 mM NaAc, pH 4.7). All glycogen and trehalose samples and standards were then resuspended and incubated at 37°C for 2 h. After centrifugation, 10 µl of the supernatant was put into a well of a 96 microtiter plate and 200 µl of GOD-PAP reagent (Dialab, Wiener Neudorf, Austria) was added. The absorbance was measured at 505 nm (Spectramax plus 384, Molecular Devices, Sunnyvale, USA). To calibrate the glucose reaction, glucose standards (0, 1, 2, 3 and 4 mM) were also measured in the same way. Glycogen and trehalose analysis of each sample was done in duplicate.

Quantitative polymerase chain reaction

The expression level of the stress related genes *HSP104*, *HSP12* and *TDH*, which is the sum of three genes *TDH1*, *TDH2* and *TDH3*, were determined using quantitative polymerase chain reaction (qPCR). Twenty-millilitre samples were collected from the tall tubes during exponential (at 40% ADF) and stationary (70% ADF) phase, cooled on ice and centrifuged at 3,000 rpm for 3 min. The pellets were washed two times with cold, sterile RNase-free water and 150×10^6 pelleted cells were then frozen at −80°C. RNA extraction was performed with Trizol (Invitrogen, Carlsbad, USA), 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, USA). Concentrations were measured and samples diluted to 100 ng/µl. The 25-µl PCR reaction mix was composed of 12.5 µl Power SYBR Green qPCR Master Mix (Applied Biosystems, Warrington, UK) and 1.25 µl of each primer (500 nM). Five microlitres of cDNA was added to each reaction mix. The PCR program used on the ABI Prism 7500 instrument (Applied Biosystems) consisted of an initial denaturation of 10 min at 95°C, amplification by 40 cycles of 15 s at 95°C and 1 min at 58°C (optimal annealing temperature of the specific primer pair). The primers were designed to anneal close to the 3′-end of each gene. The PCR primers were all designed with the PRIMER EXPRESS software (Applied Biosystems, Cheshire, UK) according to the Applied Biosystems guidelines. Primer sequences used for qPCR analysis (from 5′ to 3′): *HSP104*-F: GCGGTCTTACCGATACCTGG, *HSP104*-R: GACTGAGCAGGCTCGTCAAGG, *HSP12*-F: AGGTAGAAAAGGATTCGGTGAAA A, *HSP12*-R: GTATTCCTTACCTTGTTCAGCGTAT, *TDH*-F: CGCT TCTTGTACCAC CAACTGTT, *TDH*-R: CCGAAAGCAT CGTTGATAACC, *RDN18*-F: CGGCTACCAC ATCCA AGGAA, *RDN18*-R: GCTGGAATTACCGCGGCT. The specificity of the primers was tested using conventional PCR and the melting curves of the amplified product. The gene for 18S rRNA (*RDN18*) was used as the reference gene because the expression of this gene was found to be stable under our conditions. The expression levels were determined using the ABI Prism 7500 System Gene Quantification Software (Applied Biosystems) through quantification of Sybr Green fluorescence. A standard curve of each gene was constructed with genomic DNA. The expression levels of the different genes were normalised with respect to 18S expression levels and are means of two independent fermentation samples, each consisting of three replicates.

Acidification power test

The procedure to test the vitality of the yeast suspensions was based on Kara et al. (1988). Samples were centrifuged and resuspended in 20 ml ice-cold ultrapure water. Circa 10^8 cells were transferred in 10 ml ultrapure water (Synergy UV, Millipore, Billerica, USA) and washed three times. The

yeast suspension was transferred into a beaker filled with 20 ml ultrapure water at 25°C, of which the pH was adjusted to 6.3 with 0.01 M NaOH. During the following 20 min, the pH was measured every minute and after 10 min, 5 ml of a 20% *w*/*v* glucose solution (pH=6.3) was added to the suspension. The acidification power was calculated as the pH difference between the pH of water (6.3) and the pH after 20 min. The test was done in triplicate.

Fatty acid analysis

Total fatty acids [palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), oleic acid (C18:1) and linoleic acid (C18:2)] of frozen yeast pellets were analysed after direct saponification of 2 ml suspension with 0.1 g/ml cells in a 30-ml capped Pyrex tube with 10 ml of an equal volume mixture of 1 M potassium hydroxide and methanol at 100°C for 30 min (Moonjai et al. 2002). One millilitre of heptadecanoic acid (C17:0) (0.25 mg/ml in methanol) was used as an internal standard. After cooling, the saponified mixtures were acidified with 1 ml of HCl (6 N) followed by extraction with 3 ml *n*-hexane. After vigorous resuspension (30 s) and centrifugation (3 min, 3,000 rpm), the upper hexane layer was transferred to a 10-ml pyrex tube. A second extraction with 3 ml hexane was followed. The lipid extracts were evaporated to dryness by N_2 purging at 37°C. The methylation of fatty acids was achieved by incubation of the dried extract with 1 ml of boron trifluoride in methanol (14% solution) for 10 min at 100°C. After cooling, 6 ml of NaCl-saturated water was added and the fatty acid methyl esters were extracted with 300 µl of toluene for gas chromatographic analysis. Gas chromatography was done with a calibrated Varian 300 analyser (Varian, Palo Alto, USA) equipped with a Heliflex AT-225 capillary column (length 30 m; 0.32 mm internal diameter; layer thickness 0.25 µm; Alltech Associates, Inc., Deerfield, USA) and flame ionisation detector. Conditions were as follows: oven temperature of 75°C during 1 min, increased to 175°C at a rate of 24°C/min and 2 min at 175°C, increased to 200°C at a rate of 8°C/min and held at 200°C for 4 min; injection port at 250°C and detector at 230°C. The carrier gas was helium. Fatty acid concentrations were calculated as milligram per gram cell dry weight. The fatty acid analysis of each fermentation sample was done in duplicate.

Results

Wort fermentations were carried out in lab-scale tall tubes at 15°C, using a normal fermenting, moderately flocculating industrial lager yeast strain (CMBSPV09). Five different pitching rates (10, 20, 40, 80 and 120×10^6 cells/ml) were used to evaluate the impact of the initial cell concentration on the yeast physiology and beer quality.

Fermentation characteristics

The progress of the fermentations with different pitching rates is depicted in Fig. 1a. The time required to reach an apparent degree of fermentation of 80% was approximately 335 h for the lowest inoculum size (10×10^6 viable cells/ml), 220 h for the pitching rate of 20×10^6 viable cells/ml (this is the pitching rate used in conventional brewery fermentations), 90 h for the two-fold higher pitching rate, 50 h for the four-fold higher pitching rate and 40 h for the highest pitching condition (120×10^6 viable cells/ml). This means that, when doubling the normal pitching rate, a reduction of the fermentation time of 60% could be achieved. In case of a four-fold and a six-fold higher yeast concentration, time reductions of 78% and 82% were obtained. It is clear that the time advantage of even higher initial cell concentrations would become minor.

FAN amounts were determined throughout the fermentation and it was shown that FAN consumption was dependent on the pitching rate used. The total FAN uptake levels were enhanced with 40%, 61% and 66% when two-, four- and six-fold higher than normal pitching rates were used (Fig. 1b). For the lowest yeast concentration, a 16% lower consumption was observed.

In Fig. 1c, the growth profiles under the different conditions are shown. Because of the large differences in fermentation time, the sugar consumption (the percentage of extract consumed relative to initial extract content) was used to represent the data. As expected, the maximum yeast cell count increased with increasing pitching rate. Interestingly, the net growth (the maximum yeast cell count minus the initial inoculum size) at the three lowest pitching rates was similar (37×10^6 cells/ml for the lowest pitching rate and the reference pitching condition, and 33×10^6 cells/ml for the pitching rate of 40×10^6 cells/ml), but decreased significantly at the higher pitching rates: 24×10^6 cells/ml (condition with pitching rate of 80×10^6 cells/ml), and 12×10^6 cells/ml (for the condition with pitching rate of 120×10^6 cells/ml). Hence, lower amounts of young cells were generated in the high cell density fermentations, which could have important consequences for further generations of the yeast population (Powell et al. 2003). These results were confirmed when the total biomass (which was the sum of the yeast in suspension and the flocculated yeast formed during fermentation) was measured at the end of fermentation (Fig. 1d). From Fig. 1d, it can be concluded that at even higher pitching rates (>120×10^6 cells/ml) no growth at all will occur during the fermentation with this yeast strain and under these fermentation conditions.

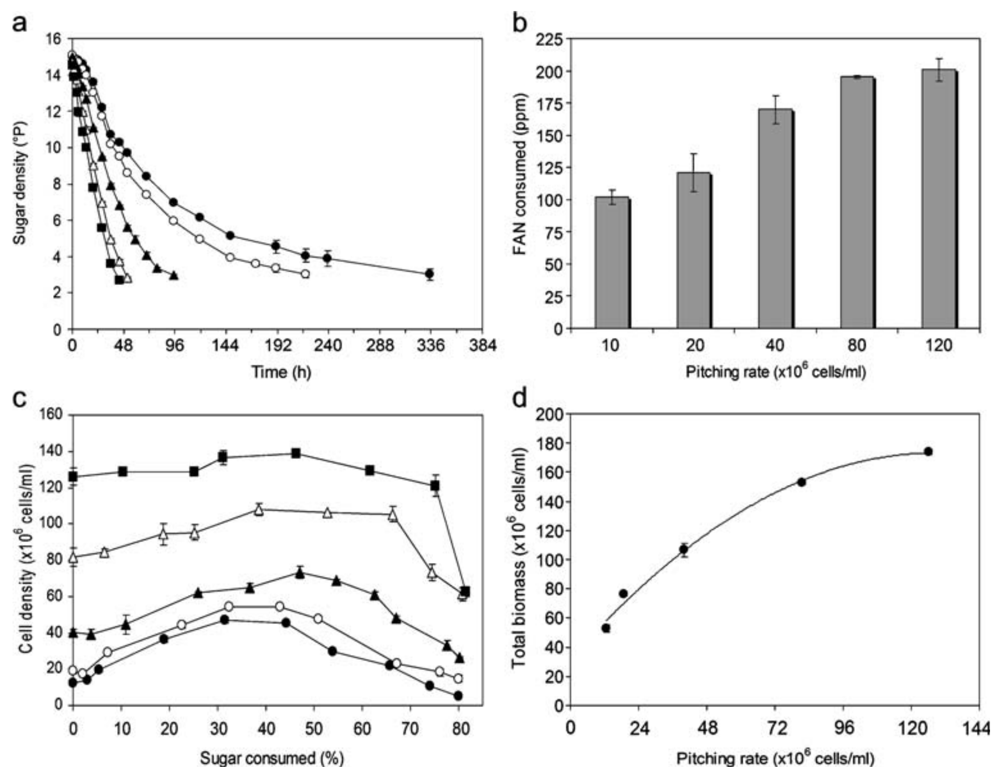

Fig. 1 Fermentation characteristics with different pitching rates. (**a**) Sugar density (expressed as °P) as a function of time, (**b**) total free amino nitrogen (*FAN*) consumed (initial FAN minus residual FAN), (**c**) cell density as a function of sugar consumed at a given time point and (**d**) the total biomass produced (measured after sedimentation of the yeast in the tall tubes, gently discarding the beer, adding 1 l cold water and vigorous shaking with the Yeastcyte apparatus) as a function of the initial pitching rate. Pitching rates: *filled circle* 10×10^6 cells/ml, *empty circle*=20×10^6 cells/ml, *filled triangle* 40×10^6 cells/ml, *empty triangle* 80×10^6 cells/ml and *filled square* 120×10^6 cells/ml

Yeast physiology

To investigate the physiology of the yeast population during and after fermentation, several parameters were studied.

Viability The viability of the yeast population was monitored during fermentation by using the fluorescent dye propidium iodide (Fig. 2). Starting with the same viable yeast population, different profiles were obtained: the three fermentations with the lowest pitching rate all maintained a high level of cell viability till about half of the sugar was consumed and decreased afterwards. For the fermentations with the highest cell density, the viability decreased gradually during the fermentation, although it also dropped faster towards the end. For these two conditions, the viability at the end was not significantly different. When the total biomass (yeast in suspension and in the precipitate) was harvested at the end of the fermentation, the cell viability for all fermentations was close to each other (mean value for all fermentations 94.2±1.2%) except for the fermentation with the lowest pitching rate where the viability was significantly lower (89.3%). This indicates that the long duration of this fermentation had a deleterious influence on the viability of the total yeast population. We can conclude that yeast viability remains at an acceptable level in all fermentations with an enhanced pitching rate.

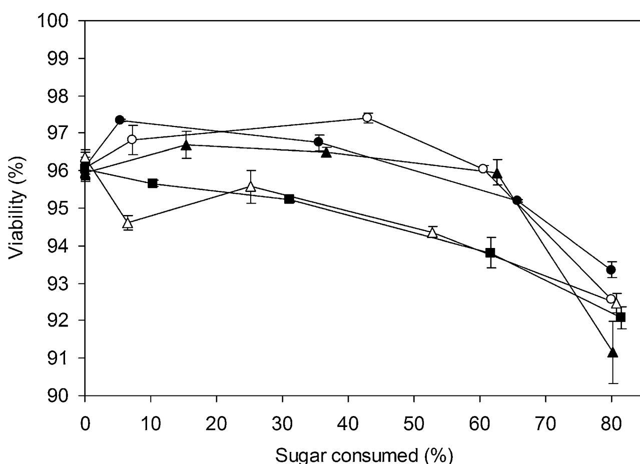

Fig. 2 Cell viability (in percent living cells) as a function of sugar consumed in the fermentations with different pitching rates. Pitching rates: *filled circle* 10×10^6 cells/ml, *empty circle* 20×10^6 cells/ml, *filled triangle* 40×10^6 cells/ml, *empty triangle* 80×10^6 cells/ml and *filled square* 120×10^6 cells/ml

Acidification power This test is a measure for yeast vitality. It depends on the endogenous carbohydrate reserves, as well as on the glycolytic activity of the yeast. At the end of the fermentation, the yeast suspensions were immediately analysed for acidification power. At the lowest cell densities (AP=1.18±0.06 and 1.13±0.06), the yeast had a significantly lower acidification power than at the higher cell densities (AP=1.53±0.02 for the pitching rate of 40×10^6 cells/ml; AP=1.41±0.03 for 80×10^6 cells/ml; and 1.42±0.06 for 120×10^6 cells/

ml). This suggests that yeast vitality was better maintained in the fermentations with higher pitching rate.

Glycogen and trehalose content Trehalose profiles largely followed the same course during the different fermentations (Fig. 3a). Initially, the cells started with a low trehalose concentration, but after the lag phase, trehalose gradually increased to reach a maximum level at the end of the fermentation. The two lowest pitching rates resulted in significantly lower trehalose accumulation in comparison with the three highest yeast densities. This was also the case for the trehalose content of the cropped yeast slurries after fermentation (data not shown). Because trehalose is known to accumulate in response to different stress conditions, these results suggest that increasing the initial cell density above a certain level may lead to more stress on the yeast. The higher trehalose levels may help to ensure yeast viability during the starvation period after fermentation is completed and also during the fermentation itself, which may lead to a reduction in fermentation time (Guldfeldt and Arneborg 1998).

The profile of the reserve carbohydrate, glycogen, differed according to the pitching condition (Fig. 3b). At the two lowest pitching rates, there was an initial period of breakdown, followed by accumulation in the exponential phase and finally breakdown again during the long stationary phase. When the pitching rate was increased, this typical profile was more levelled out: the initial breakdown, the build-up during exponential phase and the following decrease were weakened (Fig. 3b). At the two highest pitching rates, the initial breakdown was absent and glycogen content varied only little over the course of the fermentation. When the yeast mass was cropped after fermentation and the glycogen content measured, no significant difference was found between the five conditions (data not shown).

Fatty acid profile In the initial aerobic phase of the fermentation, repitched yeast has to form sufficient unsaturated fatty acids (UFA) and sterols to ensure growth (David and Kirsop 1973; O'Connor-Cox et al. 1993; Rosenfeld and Beauvoit 2003). Although fatty acid synthesis continues throughout the fermentation, UFA synthesis only takes place in the presence of oxygen by means of the desaturase Ole1 (Daum et al. 1998). In Fig. 4, the UFA/saturated fatty acid (SFA) index is shown for the five conditions in the course of the first 13 h of the fermentation. Since the wort was centrifuged, the levels of

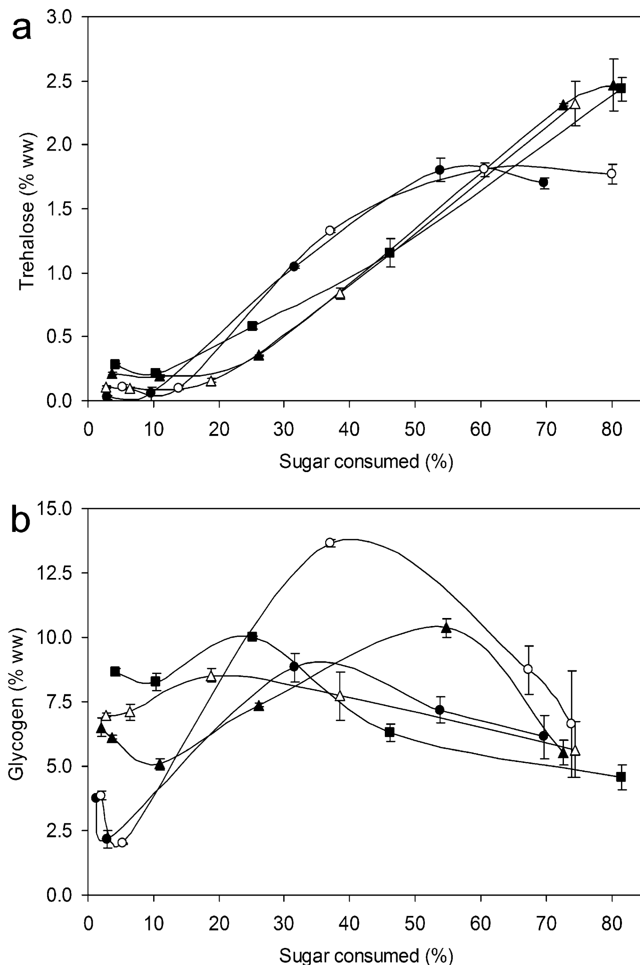

Fig. 3 Trehalose (**a**) and glycogen content (**b**) in percent of wet weight of the yeast populations as a function of sugar consumed in the fermentations with different pitching rates. Pitching rates: *filled circle* 10×10^6 cells/ml, *empty circle* 20×10^6 cells/ml, *filled triangle* 40×10^6 cells/ml, *empty triangle* 80×10^6 cells/ml and *filled square* 120×10^6 cells/ml

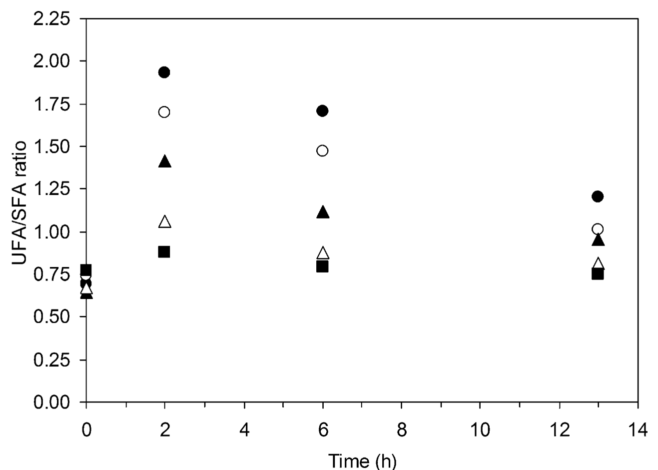

Fig. 4 UFA/SFA ratios in the yeast populations during the first hours of the fermentations with different pitching rates. Pitching rates: *filled circle* 10×10^6 cells/ml, *empty circle* 20×10^6 cells/ml, *filled triangle* 40×10^6 cells/ml, *empty triangle* 80×10^6 cells/ml and *filled square* 120×10^6 cells/ml. Maximal relative standard deviations were less than 5%

exogenous lipids were low. Hence, the strong increase in UFA content in the beginning of the fermentation must be due to biosynthesis and not to assimilation. The increase in the UFA/SFA ratio due to this increase in UFA build-up was strongly influenced by the pitching rate. The higher the pitching rate the lower the increase in the UFA/SFA ratio. Because of the strong correlation between the synthesis of UFA and yeast growth, the lower net growth at the higher cell density fermentations could be due to the smaller increase in UFA content. In the following anaerobic phase, the newly synthesised membranes are distributed over mother and daughter cells until the UFA content drops to a growth-limiting concentration (Casey et al. 1984). Indeed, the decrease in the UFA content was much steeper at the lowest pitching rates, which is consistent with the higher net growth (Fig. 4).

Expression of stress-related genes At two time points in the fermentation (corresponding to an ADF of 40% and 70%), yeast samples were taken and expression of the heat shock protein genes *HSP12* and *HSP104* was monitored by qPCR (Fig. 5). The precise function of the plasma membrane protein Hsp12 remains elusive. It has been suggested to protect the yeast plasma membrane against ethanol and desiccation toxicity (Sales et al. 2000). Hsp104 cooperates with other heat shock proteins (Hsp40 and Hsp70) to refold and reactivate damaged proteins (Glover and Lindquist 1998). In addition, global expression of the *TDH* genes was also assessed (Fig. 5). *TDH1*, *TDH2* and *TDH3* encode related isozymes of glyceraldehyde-3-phosphate dehydrogenase, which catalyses the reaction of glyceraldehyde-3-phosphate to 1,3-bis-phosphoglycerate in glycolysis.

The expression of *HSP104* was much lower than the expression of *HSP12*. It showed a slight tendency to increase with higher pitching rate in both the exponential and stationary phases. This was also observed for the expression of *HSP12* in exponential phase. In the stationary phase, on the other hand, expression of *HSP12* strongly increased at the higher pitching rates.

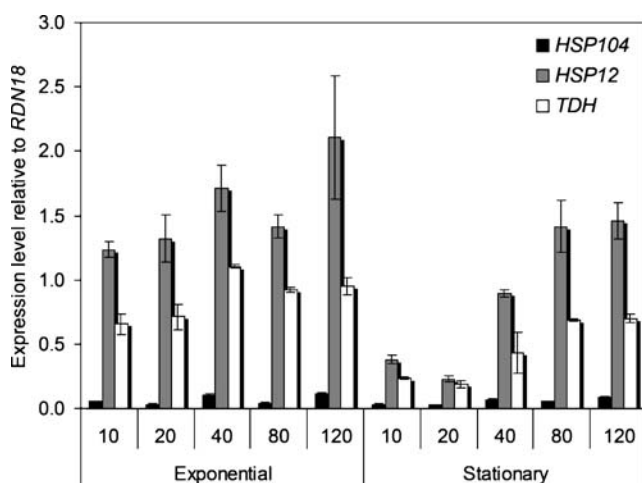

Fig. 5 Expression profiles of the genes *HSP104*, *HSP12* and the *TDH* family at 40% ADF (exponential phase) and 70% ADF (stationary phase) as a function of the pitching conditions. The levels are expressed relative to that of the housekeeping gene *RDN18*

TDH expression as a function of pitching rate was quite similar to that of *HSP12*, although the increase at the higher pitching rates in the stationary phase was not as pronounced as with *HSP12*. In the stationary phase, the transcript levels were also lower than in the exponential phase. In a study by James et al. (2003), the *TDH* genes were all repressed in the stationary phase as well. The slightly higher induction of *TDH* activity with higher pitching rates could indicate that the glycolytic activity was little increased, which was confirmed by the slightly higher acidification power of the yeast suspensions of these fermentation conditions at the end of fermentation. Higher *TDH* expression levels may correlate with higher glycolytic flux capacity (Smits et al. 2000) and are therefore consistent with the higher acidification power of the yeast harvested at the end of the fermentations with the higher pitching rates.

Beer characteristics

Table 1 shows the results of the beer analysis for final alcohol, glycerol, bitterness, pH and aroma concentrations in the fermentations performed at the different pitching rates. Despite the large differences in net growth, the yields of ethanol were identical ($Y_{p/s}$=0.558±0.001% (v/v)/°P). Also the glycerol level was not strongly different, although a slight increase was observed with increasing pitching rate. The pH of the beer was also lower when higher pitching rates were used. The pH drop during wort fermentation is caused by the assimilation of buffering compounds such as amino acids and primary phosphates, the formation of organic acids and CO_2 and the secretion of H^+ by the yeast (Boulton and Quain 2001). Therefore, the larger FAN consumption rates and the more vigorous fermentations in the case of higher inoculum size are consistent with the lower pH in the final beer.

Bitterness of the beer is an important quality parameter. It is caused by the α-acids of added hops, which are isomerised during wort boiling. The fermentation process has an important influence on the residual concentration in beer because these acids bind to the yeast cell wall (Boulton and Quain 2001). Losses in the range of 5–20% are generally observed (Laws et al. 1972). In spite of the strong differences in cell density and cell growth, no influence of the pitching rate on the final beer bitterness was observed (Table 1).

With respect to the aroma compounds, the concentration of isobutanol and isoamyl alcohol increased with higher

Table 1 Final alcohol, glycerol, bitterness, pH and aroma concentrations in the fermentations performed at different pitching rates

	Pitching rate (×10^6 cells/ml)				
	10	20	40	80	120
Alcohol (% *v*/*v*)	6.77±0.18	6.69±0.06	6.61±0.03	6.61±0.01	6.68±0.00
Glycerol (g/l)	1.70±0.05	1.75±0.12	2.02±0.02	2.01±0.28	2.20±0.08
pH	4.45±0.01	4.46±0.02	4.30±0.01	4.20±0.01	4.24±0.01
Bitterness (EBU)	21.8±0.1	20.7±1.6	20.4±0.3	20.5±1.3	24.5±0.3
Flavour profile					
Acetaldehyde (ppm)	6.48±0.97	5.94±0.63	7.45±0.38	8.66±0.28	7.21±0.24
DMS (ppb)	31.0±1.3	26.8±1.7	25.5±0.2	24.0±1.6	25.1±2.6
Higher alcohols (ppm)					
Propanol	10.7±0.2	11.0±0.3	11.3±0.1	10.6±0.1	10.9±0.2
Isobutanol	6.51±0.15	6.97±0.11	7.40±0.10	7.93±0.07	8.24±0.04
Isoamyl alcohol	45.9±1.9	48.5±0.8	54.2±0.2	58.7±0.4	57.9±0.2
Esters (ppm)					
Ethyl acetate	25.5±1.7	25.0±1.3	23.7±0.2	21.9±0.3	23.8±0.5
Isoamyl acetate	1.31±0.04	1.48±0.07	1.68±0.03	1.54±0.01	1.46±0.04
Ethyl caproate	0.15±0.01	0.15±0.00	0.17±0.01	0.18±0.00	0.16±0.00
Diacetyl (ppb)	19.1±0.1	48.4±0.7	307±6	511±5	428±3

Values of all the flavour compounds have been recalculated based on the normal ethanol percentage in lager beers (=5% *v*/*v*)

pitching rate (Table 1). In general, the concentration of higher alcohols is positively influenced by the pitching rate (Edelen et al. 1996; Verbelen et al. 2008).

Esters are important flavour compounds in lager beers because they often reach their threshold value. Ester levels have previously been reported to be negatively influenced by higher pitching rates (Suihko et al. 1993; Edelen et al. 1996; Verbelen et al. 2008). This was not observed in our experiments and any difference between the fermentations with different pitching rates was small. Slightly less ethyl acetate, which has a solvent-like flavour (threshold 30 ppm), was formed during the fermentations with higher inoculum size. In the case of isoamyl acetate (banana-like flavour, threshold 1.2 ppm), a maximum level was found at a pitching rate of 40×10^6 cells/ml; for ethyl caproate (apple-like flavour, threshold 0.21 ppm), no clear trend was observed.

DMS levels were slightly lower with increasing pitching rate. In contrast, the acetaldehyde concentrations at the end of fermentation showed a small increasing trend with increasing pitching rate.

The vicinal diketone, diacetyl, can cause a 'buttery' off-flavour above its threshold (80 ppb; Wainwright 1973). Diacetyl is formed by an extracellular oxidative decarboxylation of α-acetolactate, an intermediate in the biosynthesis pathway of valine from pyruvate. Subsequently, yeast cells assimilate and reduce diacetyl to the flavour inactive compounds acetoin and 2,3-butanediol. Diacetyl was present in dramatically higher amounts at the end of the fermentations with higher cell density (Table 1, Fig. 6). From the course of the diacetyl level during the fermentation, it can be seen that the initial increase in total diacetyl is very similar for all pitching rates. However, when about 50% of the sugar was consumed, the total diacetyl level showed a steep decline at the two lowest pitching rates whereas it continued to increase at the three highest pitching rates. Only at the very end of the fermentation when 70–80% of the sugar was consumed, the total diacetyl content also started to decrease in the fermentations with the highest pitching rate. Final diacetyl content (Fig. 6) therefore clearly correlated with the fermentation time (Fig. 1a). Because the chemical decarboxylation step of

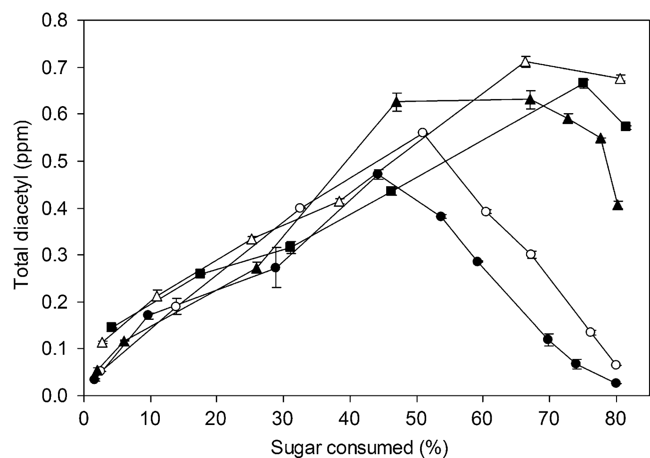

Fig. 6 Profile of the total diacetyl content as a function of the sugar consumed in the fermentations with different pitching rates. Pitching rates: *filled circle* 10×10^6 cells/ml, *empty circle* 20×10^6 cells/ml, *filled triangle* 40×10^6 cells/ml, *empty triangle* 80×10^6 cells/ml and *filled square* 120×10^6 cells/ml

α-acetolactate is the rate-limiting step in the metabolism of diacetyl, the short fermentation times in the case of the higher cell concentrations likely resulted in incomplete conversion of α-acetolactate to diacetyl, thereby preventing the subsequent reduction which normally can occur at a fast rate.

Discussion

Higher initial cell concentrations increase the fermentation speed, which creates a large potential economical advantage for the breweries. Therefore, the purpose of this study was to determine the impact of pitching rate on yeast fermentation performance and beer quality.

The impact of pitching rate on fermentation power

The initial cell concentration had a significant impact on the fermentation power (°P/day). The fermentation power was defined as the ratio of the extract decrease from 12 to 6 °P and the time (in days) in which this decrease occurred. The fermentation power increased from 1.6 to 2.0, 4.5, 6.7 and 7.3 °P/day when the pitching rate was enhanced from 10 to 20, 40, 80 and 120×10^{6} cells/ml, respectively. When looking at the specific fermentation power, no difference was observed between the different pitching rates. However, the small increases in the acidification power and the induction of *TDH* expression suggest a higher yeast activity with higher pitching rate. Overall, the differences in the yeast fermentation performance observed between the pitching conditions were small. The net growth rate followed a decreasing trend as a function of the pitching rate. This is of importance when the yeast slurry has to be reused in subsequent fermentations. Aged yeast cells often show decreased fermentation capacity, changed morphology, altered flocculation patterns and extended generation times (Smart 1999; Powell et al. 2000). Therefore, the influence of high cell density on the fermentation performance in subsequent yeast generations in successive brews will also have to be determined in the future.

Net growth is related to the build-up of essential membrane lipids during the initial aerobic phase of the fermentation (Boulton and Quain 2001). When the initial cell suspension was increased from 80 to 120×10^{6} cells/ml, the fermentation power was only slightly further increased (from 6.7 to 7.3 °P/day) whereas the net growth was halved. Therefore, it seems that an optimum cell density can be selected, which allows both a significant reduction in time and an acceptable extent of growth, corresponding to the brewer's preferences. This specific pitching rate is probably strain-specific and dependent on the oxygen requirements of the yeast. In addition, process parameters, such as wort composition and oxygen condition, can be optimised for a specific cell density.

Impact of pitching rate on yeast stress response

Although the glycogen profiles, trehalose accumulation and expression of stress genes varied with initial inoculum size, it cannot be stated that the yeast activity (i.e. the acidification power) is negatively influenced by the increase in cell suspension. *HSP12* and *HSP104* expression and trehalose accumulation appear to indicate a higher stress response of the yeast when the cell density in the fermentation medium increases. Surprisingly, the expression level of both heat shock genes was generally lower in the stationary phase than in the exponential phase. Both genes contain STRE sequences in their promoter and are induced in a variety of stress conditions, such as exposure to heat, high osmolarity and high ethanol levels, and also upon entry in stationary phase (Boy-Marcotte et al. 1999; Amorós and Estruch 2001). Therefore, a higher expression of heat shock genes at stationary phase would normally be expected. However, in a whole genome study, performed by James et al. (2003), with a similar set-up as in our experiments (pitching rate 15×10^{6} cells/ml), *HSP12* and *HSP104* expression levels were also repressed in the stationary phase. Rautio et al. (2007) observed an increase of *HSP104* and *TPS1* upon glucose depletion after the exponential phase, after which they returned to their initial expression level. In another study, performed by Brosnan et al. (2000), *HSP104* expression was shown to be strain dependent and large differences were revealed between lager strains and lab strains. Moreover, the expression levels of *HSP104* were extremely low at the end of fermentation. These authors suggested that the decrease of expression of *HSP104*, as the fermentation proceeded, could be explained by the many differences between brewery fermentations and aerobic growth in glucose medium (YPD), in which *HSP12* and *HSP104* are reported to be induced in the stationary phase. In the former, the stationary phase is a result of a lack of lipids and thus oxygen, and not of fermentable carbohydrates. In contrast, in aerobic conditions, the limiting nutrients are fermentable carbohydrates, resulting in diauxic shift and subsequent respiratory growth. Moreover, each yeast strain has its own particular level of expression of *HSP12* and *HSP104* (Carrasco et al. 2001; Garay-Arroyo et al. 2004). These differences and the fact that we used an industrial yeast strain, instead of a laboratory strain, can explain the lower expression level at the stationary phase.

On the other hand, it remains unclear to what extent the stress response influences yeast physiology and which type of stress the yeast is really exposed to. Temperatures, osmotic conditions, nutritional state of the wort and ethanol

production were the same in the different fermentations. Therefore, other stress factors must be responsible for the observed stress response. A first possibility is the depletion of an essential nutrient in the fermenting medium because of the more intense fermentations. In addition, the decrease in pH could have caused the stress response. A third possibility is a molecular response due to the high cell concentrations itself. Chen and Fink (2006) have shown that aromatic alcohols, which are only produced at high cell densities, induce morphological changes in *S. cerevisiae*. The quorum sensing phenomenon (the expression of a specific phenotype as a response to small cell density-dependent signal molecules) in *S. cerevisiae* yeasts is still poorly explored. In higher eukaryotes, the cell density is well known to have important regulatory functions on the growth of cells. By means of cell–cell contact, growing cells sense whether to continue or arrest growth and multiplication. At high cell density, adequate cell–cell contacts are established whereby growth arrests. Although similar growth regulatory mechanisms have been observed in *S. cerevisiae* cultures where high cell densities caused early growth arrest of non-*S. cerevisiae* cells (Nissen et al. 2003), the significance of quorum sensing and cell–cell contact-mediated functions in industrial fermentations is not clear. Whole genome studies and proteomics could help to unravel the physiological state of the yeast cell as influenced by high cell densities.

Impact of pitching rate on beer quality

An important prerequisite for the successful application of higher initial cell concentrations is the maintenance of beer quality. In contrast with other parameters (oxygen, temperature, agitation; Sablayrolles 1995; Sasaki et al. 2000; Boswell et al. 2002), the impact of pitching rate on flavour formation seems to be minor in this study, with the exception of diacetyl. The synthesis of higher alcohols is directly related to amino acid metabolism (i.e. Ehrlich pathway), which is in turn related to cell growth (Hazelwood et al. 2008). Therefore, higher uptake rates of the amino acids can result in higher concentrations of the corresponding higher alcohols. In our experiments, however, we observed lower net growth rates at higher initial cell concentrations, and thus the higher alcohol production was not influenced by growth, but more probable by the stronger fermentation power at these higher cell densities.

In many accelerated beer fermentations systems, high levels of total diacetyl in the final beer are known to be caused by a higher production of its precursor α-acetolactate and/or the incomplete chemical conversion of α-acetolactate to diacetyl. In the latter case, incomplete removal of diacetyl by the yeast occurs, due to the short residence times (Okabe et al. 1992; Brányik et al. 2005; Willaert and Nedovic 2006; Verbelen et al. 2008). Several strategies can be followed to decrease the diacetyl content in those beers, such as accelerated maturation using immobilised yeast, supplementation of α-acetolactate decarboxylase to the wort or the use of genetically modified yeast strains (Hammond 1995; Linko et al. 1998; Hanneman 2002).

Taken together, this work provides evidence that significant time savings in the fermentation process are possible by increasing the pitching rate. Limited physiological and metabolic differences were observed in the yeast cells at higher cell densities, suggesting that the yeast population was not negatively influenced. On the other hand, diacetyl levels were strongly enhanced, which will require further optimisation of the yeast or the process conditions. Also, more experimental work is needed to evaluate the stability of high cell density yeast populations in subsequent generations, the role of oxygen conditions during high cell density fermentations and the technological feasibility at a commercial scale.

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