Research article

Institute of Brewing & Distilling

Received: 3 February 2014 Revised: 25 July 2014 Accepted: 26 July 2014 Published online in Wiley Online Library: 29 September 2014

(wileyonlinelibrary.com) DOI 10.1002/jib.171

High gravity primary continuous beer fermentation using flocculent yeast biomass

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The current work assessed a new immobilized cell reactor system throughout a long-term (54 days) continuous primary fermentation of lager-type wort of high specific gravity. The experiment was performed in a 4 L airlift bioreactor and immobilization of biomass was attained solely by flocculation. Despite the constant liquid agitation and washout of biomass, up to 53 g dry wt/L of yeast remained immobilized in the system. Two types of beer were produced without interrupting the reactor, based on two types of wort: a Pilsener type with high specific gravity of 15.6 ± 0.3°P; and a dark lager wort with specific gravity of 14.4 ± 0.03°P. Even during the inlet of high gravity wort, the desired attenuation was achieved without the need for either recirculation or an auxiliary second stage bioreactor. The specific saccharide consumption rate was kept around 7.9 ± 0.4 g/L/h and ethanol productivity oscillated at 3.36 ± 0.2 g/L/h for nearly a month. During this period the volumetric productivity of the current bioreactor reached 1.6 L beer/L/day. The green beers produced from the Pilsener and dark lager worts met the standards of regular finished primary beer fermentation. The productivity of diacetyl through the entire experiment could be correlated to the free amino nitrogen consumption rate.

Keywords: yeast flocculation; continuous beer fermentation; volumetric productivity; diacetyl accumulation

Introduction

High-gravity brewing (HGB) and continuous fermentation are two well-known options for increasing the productivity of a brewery. However, while HGB is a fully implemented and quite reliable technology, continuous fermentation is still avoided by most breweries. A major reason for this is because the latter requires additional investments in equipment, which strongly modifies the process as a whole. Additionally, continuous beer fermentation still needs fine-tuning concerning technical *(1,2)* and sensorial *(3,4)* problems affecting the final product. Nonetheless, considering that continuous fermentation can greatly increase volumetric productivity, the investment cost could be justified if a reliable and efficient setup was developed.

Any continuous fermentation system depends on high concentrations of yeast, often attained by immobilized cell technology (ICT). Two methods are usually applied in ICT bioreactors: attachment of yeast to solid carriers for biofilm formation *(5–8)*; and forced physical entrapment of yeast cells into semi-rigid matrices *(9–12)*. For both of these methods the yeast carrier price often limits further applications of the technology. Flocculation on the other hand is a natural (free of charge) method of immobilization, where yeast cells attach to each other to form large cell aggregates – flocs. Flocculation is a multifactorial inheritance triggered by both genetic *(13,14)* and environmental factors *(15–17)*, being widely used to harvest yeast at the end of primary fermentation in traditional brewing *(18,19)*.

Airlift bioreactors are remarkably efficient in mass transfer of either liquid–liquid or solid–liquid phases triggered by rapid mixing, low shear stress, simple design and low energy consumption *(20)*. These attributes give airlifts excellent conditions for application in conjunction with ICT, which has previously been tested under continuous beer production *(4,6,11,12)*.

Relying only on yeast flocculation as an immobilization method can be challenging as self-aggregation is hard to predict *(15,16,19)*. This task becomes even harder when the input wort has a high specific gravity (SG) because the inlet sugars will competitively bind to yeast's flocculins, preventing these proteins from attaching to mannose residues of surrounding cells, thus inhibiting flocculation *(16,17,19)*. Additionally, an extended continuous fermentation may also compromise yeast viability owing to yeast's limited lifespan *(21,22)* and the restricted time of metabolic activity *(23,24)* of the immobilized cells. Furthermore, when mechanical agitation is absent (as in batch fermentations), dead cells are removed from the bottom cone of the cylindroconical fermenters by preferential sedimentation of the older cells *(18,25,26)*, which is not compatible with the constant agitation of an airlift reactor. Although ambitious, the possible synergy offered by placing together HGB and continuous fermentation could greatly increase process productivity.

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Therefore, this work assessed the feasibility of a long-term continuous HGB in an airlift bioreactor, using flocculation alone for biomass immobilization. Key parameters such as yeast viability and the chemical composition of the green beer were evaluated in detail through the course of the fermentation. Maturation was performed in bottles to assess the final quality of the beer.

Methods

Brewing yeast

The flocculent *Saccharomyces pastorianus* strain no. 96 from the culture collection of the Research Institute of Brewing and Malting Plc. (Prague, Czech Republic) was used. Yeast was isolated in a Petri dish containing yeast extract peptone dextrose broth (Sigma-Aldrich, UK) with 2% wt of agar (Sigma-Aldrich, UK). A few colonies were then inoculated into 400 mL of 15°P wort and incubated at 30°C, 120 rpm, for 48 h prior to being used as inoculum for the gas-lift reactor.

Beer wort

Wort used in this experiment was supplied by UNICER Bebidas, S.A. (S. Mamede de Infesta, Portugal). It was collected from the main production line after the cooling stage and, prior to aeration, stored in 50 L barrels (Nalgene, USA), taken to the laboratory, heated to 100°C and autoclaved for 1.5 h at 121°C. Considering the high volume of the barrels, heat treatment is mandatory for keeping the wort free of contamination during long-term experiments. The composition of each barrel of wort supplied to the reactor is presented in Table 1.

Continuous fermentation

The continuous fermentation was carried out in a Perspex airlift bioreactor with 4 L of total work volume. Gas flow was regulated by two independent mass controllers (Alicat Scientific, USA) using CO_2 (100 mL/min) and pressurized (2 bar) air (10 mL/min). This mixture and flow were kept constant in the course of the entire experiment. The temperature was held at 15°C using a Julabo F32 Refrigerating/Heating Circulator (Julabo, Germany). The dilution rate was controlled by a peristaltic pump 101U/R (Watson-Marlow, UK). Fig. 1 shows an overview of the setup used in this work.

The reactor was sterilized using a 3% (v/v) solution of commercial sodium hypochlorite with 1.5% of active chlorine, 48 h before use. After this time, the solution was discarded and 50 L of sterile water was used to wash the reactor. It was then filled with 15°P Pilsener wort and inoculated.

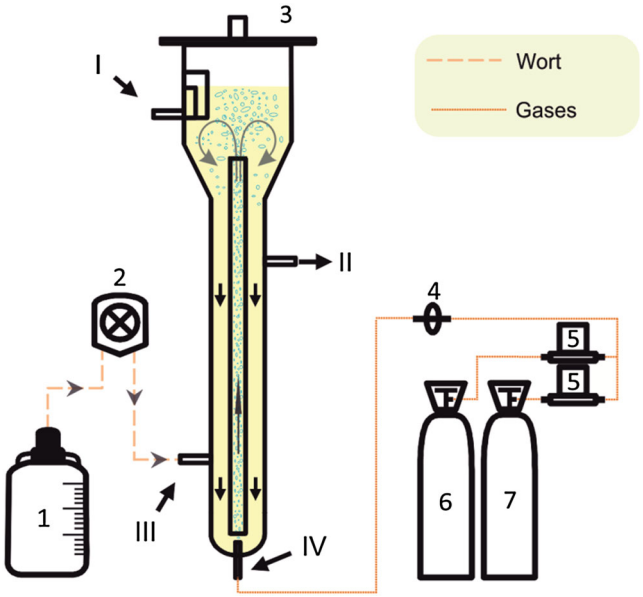

Figure 1. Immobilized yeast bioreactor system used in the current work: 1, wort barrel; 2, peristaltic pump; 3, gas-lift reactor; 4, air filter; 5, flow mass controllers; 6, pressurized air; 7, CO_2 bottle; I, green beer outflow/sampling point; II, biomass sampling point; III, wort inlet; IV, gas sparger. This figure is available in colour online at wileyonlinelibrary.com/journal/brewing.

The reactor operated in batch mode for the first 48 h at 20°C, using only pressurized air as the gas supply at 200 mL/min. After that, air-flow was reduced to 10 mL/min at the same time that CO_2 injection was started at 100 mL/min. Afterwards, the continuous mode of operation was started with the wort barrel no. 1 (Table 1) at a dilution rate of 0.019/h. Subsequently, the dilution rate was raised gradually (up to 0.066/h) depending on the attenuation level of the green beer.

Biomass measurements

The flocculated biomass was evaluated daily. Three 15 mL plastic Falcon tubes were dried at 105°C for 12 h and weighed. Then 10 mL of reactor volume was taken at the sampling point II (Fig. 1) and added to each Falcon tube and centrifuged at 4000***g*** for 5 min. The liquid phase was discarded and the tubes were dried (105°C) for 24 h prior to weighing. The same procedure was also carried out with samples of green beer directly from the outflow (Fig. 1I)

Table 1. Composition of inlet wort from the barrels

(Barrel no.) Wort type	Period in days (from–to)	RT_{tot} (h)	SG (°P)	pH	Colour (EBC)	EBU	FAN (mg/L)
(1) Pilsener	0–11	59–27	15.95	4.91	26.5	25	203
(2) Pilsener	11–20	20	15.67	4.79	26.0	27	178
(3) Pilsener	20–27	17	14.98	4.82	20.6	26	176
(4) Pilsener	27–34	15	15.76	4.74	31.6	34	209
(5) Pilsener	34–40	15	15.50	4.76	28.1	33	207
(6) Dark lager	40–48	15	14.43	4.77	224	19	183
(7) Dark lager	48–54	15	14.40	4.75	219	20	191

SG, Specific Gravity; RT_{tot}, Retention Time;°P, Plato degree; EBC, European Brewery Convention; EBU, European Bitterness Units; FAN, Free Amino Nitrogen.

in order to evaluate the loss of biomass. Blank experiments were carried out using the inlet wort to correct for the presence of trub-like compounds that could interfere with weighing. After corrections, the dry matter was considered as the total biomass (X_{tot}), corresponding to both flocculated and free cells suspended inside the reactor.

Yeast viability

Yeast viability, from either X_{tot} or cells from the outflow, was assessed by flow cytometric measurements. Stock solutions of fluorescein diacetate (FDA) and propidium iodide (PI) were prepared. FDA (Sigma, UK) was diluted with dimethyl sulphoxide (Sigma, UK) to a final concentration of 500 μg/mL. PI (Sigma, UK) solution was diluted in ultra-pure water to a final concentration of 1 mg/mL. These stock solutions were kept at −20°C until used. For the double staining with FDA and PI, 100 μL of ~1×10^6 cell suspension was added to 400 μL of phosphate-buffered saline (Sigma, UK) containing 1 μL of PI stock solution and 4 μL of FDA. The incubation was carried out at room temperature for 20 min.

Samples were double-stained with FDA and PI as described above, and analysed using a Beckman-Coulter EPICS XL flow cytometer (Beckman Coulter, Inc., USA) equipped with an Argon ion air-cooled laser (emission at 488 nm/15 mW power). The FDA green fluorescence was detected by the FL1 (530 nm) channel, while the PI red fluorescence was captured by the FL3 (590–610 nm). More than 20,000 cell readings were carried out for each sample (in triplicate). Data were analysed using Flowing Software Version 2.5.1 (Freeware provided by Perttu Terho, Turku Centre for Biotechnology, University of Turku, Finland).

Beer maturation

Green beer from the bioreactor outflow was collected in a closed plastic recipient vessel, immersed in ice. Then it was filled into 1.5 L PET bottles, containing 50 mL of the respective original wort for carbonation and kept for 30 days at 6°C.

Beer and wort analysis

Characterization of the wort, green and maturated beer was performed as suggested in the most recent version of Analytica EBC methods. All reagents were purchased from Merck (Germany).

Periodically, the green beer taken from the outflow was filtered using a vacuum filtration apparatus (New Star Environmental, USA) coupled to a filtering flask immersed in ice. Cellulose nitrate membranes, with a 0.2 μm pore size (Whatman, USA), were used for filtration. Filtered samples were then placed into 50 mL Falcon tubes and frozen (−20°C) until further analyses were performed.

Ethanol concentration, specific gravity, original and real extract, pH, colour and degree of fermentation were determined using the automatic beer analyser Anton Paar Beer Analysis System DMA 4500 (Anton Paar GmbH, Austria). Total diacetyl was determined by gas chromatographic analysis of the static headspace using a Varian CP-3800 Gas Chromatograph (Varian, USA). Separation was carried out by a wall-coated open tubular fused silica capillary column (60 m × 0.25 mm) CP-Sil 8 CB low bleed/ms (Varian, The Netherlands) using nitrogen as carrier gas. Samples were automatically injected by a Combi PAL headspace autosampler (CTC Analytics, Switzerland).

Acetaldehyde, higher alcohols and esters were measured by a Varian Star 340 CX Gas Chromatograph (Varian, USA) with a flame ionization detector and a thermoconductivity detector. The volatiles were separated by a wall-coated open tubular fused silica capillary column (60 m × 0.53 mm) CP-Wax 52 CB (Varian, The Netherlands) using pure air and hydrogen as carrier gases. Samples were automatically injected using a Combi PAL headspace auto sampler (CTC Analytics, Switzerland). The free-amino nitrogen (FAN) and bitterness results were measured in a Varian Spectrophotometer Cary 50 Bio UV-Visible (Varian, USA).

Results and discussion

Yeast growth and viability

As Fig. 2 shows, X_{tot} had a consistent tendency to accumulate in the airlift reactor in the course of all continuous fermentation experiments. It is important to emphasize that other works involving ICT normally distinguish between immobilized and free cells, where the sum of these two fractions is equal to the X_{tot} used in this work.

In order to build the needed X_{tot} to achieve high productivities, the wort was initially fed to the airlift at a very slow flow rate (Q = 0.074 L/h). As a consequence, the total retention time (RT_{tot}) was 59 h, but before completing the first RT_{tot} cycle (48 h) the X_{tot} was already 14.7 $g_{cell\ dry\ wt}$/L. As the current system relied on flocculation, the tendency for X_{tot} accumulation inside the airlift depended on the biomass production rate (*BPR*) and biomass washout rate (*BWR*). *BPR* was defined as the capacity of biomass production by X_{tot} in a given time, calculated as follows:

$$BPR = X_{tot} * \mu$$

BWR was evaluated experimentally by constant measurements of biomass concentration in the outflow (X_{out}) of the airlift reactor and can be expressed as:

$$BWR = X_{out} * D$$

Theoretically, if the values of *BPR* and *BWR* were equal, X_{tot} would have been kept constant. Specific growth rate (μ) has been calculated based in experimental data from this experiment at different stages of the continuous fermentation, as follows:

$$\mu = \frac{\Delta X}{\Delta t_{(1-0)}} * \frac{1}{X_{tot_{t0}}}$$

Considering that biomass was constantly being removed from the reactor through the outflow, ΔX in the current system may be represented by:

$$\Delta X = (X_{tot_{t1}} - X_{tot_{t0}}) + X_w$$

where X_w is the total biomass concentration removed from the system during Δt. Thus:

$$\mu = \frac{(X_{tot_{t1}} - X_{tot_{t0}}) + X_w}{\Delta t_{(1-0)}} * \frac{1}{X_{tot_{t0}}}$$

The maximum specific growth rate, μ_{max}, was observed in the first days of continuous operation and was around 0.013/h, with the average value throughout the experiment being around 0.008/h. Moreover, it was evidenced (Fig. 2) that *BWR* oscillated below *BPR* in a large part of the continuous experiment, which

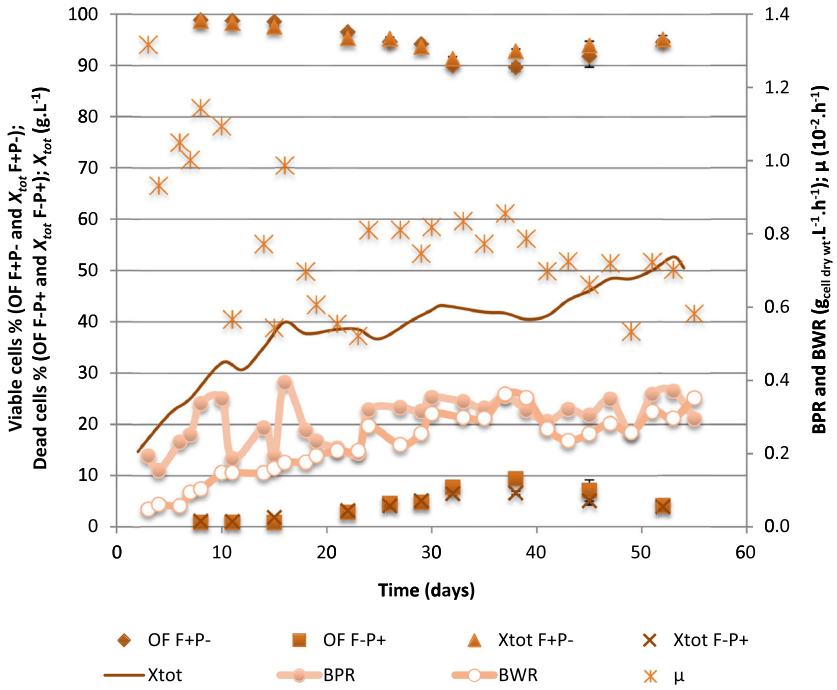

Figure 2. Biomass specific growth rate (μ), biomass productivity rate (*BPR*), biomass washout rate (*BWR*) and yeast viability assessed by flow cytometry during the continuous fermentation. *OF* F+P−, Viable cells from outflow (Fig. 1I) with positive staining for fluorescein diacetate and negative staining for propidium iodide; X_{tot} F+P−, viable cells from sampling point II (Fig. 1) representing the X_{tot} with positive staining for fluorescein diacetate and negative staining for propidium iodide; *OF* F−P+, dead cells from outflow (Fig. 1I) with positive staining for propidium iodide and negatively stained by fluorescein diacetate; X_{tot} F−P+, dead cells from sampling point II (Fig. 1) representing the X_{tot} with positive staining for propidium iodide and negatively stained by fluorescein diacetate. The viability values presented in this figure are an average of 20,000 cell readings, carried out in triplicate. This figure is available in colour online at wileyonlinelibrary.com/journal/brewing.

led to the positive tendency of X_{tot} retention by flocculation. This trend was observed during major part of the experiment, except in the period between the days 27 and 40 of continuous fermentation, when *BWR* began to rise reaching its peak above *BPR* on day 39, which was reflected in a small period of X_{tot} decrease. This period matches the increase in the saccharide loading rate (*SLR*) and the free amino nitrogen loading rate (*FLR*) caused by both changing wort barrels and by rising *Q* (Fig. 3A,B). Flocculation is affected by a combination of many variables, one of the most important being the availability of nutrients (e.g. sugars and FAN) *(19)*.

Intriguingly, the same increase in *SLR* and *FLR* reported in the last lines also decreased X_{tot} viability (Fig. 2), which showed signs of recovery when *SLR* and *FLR* were reduced. This loss of viability may be correlated with the impairment of the stress response caused by the increased availability of fermentable sugars (triggering the Ras–cAMP–PKA pathway) and FAN (activating the target of the rapamycin–TOR pathway) *(27)*. Yeast viability was assessed by esterase activity and preservation of plasma membrane integrity monitored by FDA and PI, respectively.

The maximum value of X_{tot} measured in the current work was 52.7 g dry wt/L, a number far greater than other ICT setups, including either entrapment-based *(8)* or biofilm-based *(4,7)* systems. However, the amount of yeast associated with a specific system does not necessarily reflect good results. Therefore, the performances of different ICT setups are often compared in terms of specific saccharide consumption (r_s) and volumetric ethanol productivity (r_p), as discussed in the next section.

SLR, *FLR*, nutrient consumption and productivities

The performance of the bioreactor depends not only on its ability to assimilate what it is being fed, but also on the yield of the desired product. Fig. 3 drafts the entire course of *SLR* and *FLR* fed to the airlift reactor, their respective consumption rate and the productivity values of the most important components of green beer – ethanol, higher alcohols and esters.

Coupling continuous fermentation with HGB, associated with short RT_{tot}, resulted in r_s values as high as 8.7 g/L/h and r_p of ethanol reaching 3.7 g/L/h. Moreover, none of these values were obtained with loss of attenuation performance (Table 2). In fact, the values observed in the current work are far greater than any other published data for continuous beer fermentation *(4,6,8,11,12)*. Additionally, as long as RT_{tot} was fixed at 15 h, r_s and ethanol r_p remained steady within 7.9 ± 0.4 and 3.36 ± 0.2, respectively, for nearly a month until interruption of the fermentation (Fig. 3A). In other words, during all stages (including the highest productivities RT_{tot} = 15 h), the desired attenuation was reached, which means a complete primary beer fermentation. Throughout this period the volumetric productivity of the reactor was 1.6 L beer/L/day. This means that a 4 L reactor was producing 6.4 L of green beer per day.

Although high osmolarity has been targeted as being one of the main stress factors during HGB *(28,29)*, ethanol tolerance developed by lager yeast strains was recently observed to increase fermentation rates *(30)*. As fermentation in this work ran in a continuous mode, sugar levels inside the airlift reactor were low owing to the quick uptake of nutrients by the large amount of X_{tot} in the reactor; on the other hand the ethanol concentration was always around 52 ± 3 g/L. Although (together with other stress factors) the ethanol stress might have discouraged biomass growth (μ_{max} = 0.013/h), evidence reported by Hasegawa *et al*. *(30)* may support the good performance of fermentation rates for the current setup.

In contrast to what is observed in the ratio of SLR/r_s, which remained quite stable during the experiment, the free amino

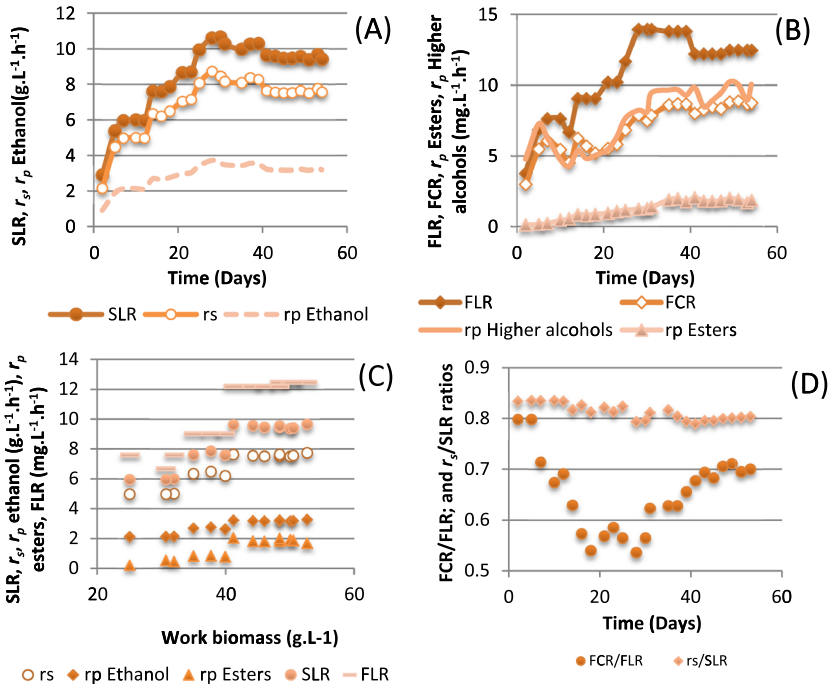

Figure 3. Organic loading/consumption rate of nutrients present in the feed wort and productivity rates of main components measured in the green beer from the outflow during the continuous fermentation. (A) Saccharide loading rate (*SLR*), saccharide consumption rate (r_s) and ethanol productivity (r_p ethanol), in the course of continuous fermentation time. (B) Free amino nitrogen loading rate (*FLR*), free amino nitrogen consumption rate (*FCR*); total higher alcohols productivity (r_p higher alcohols) and total acetate esters productivity (r_p esters), through fermentation time. (C) Consumption and productivity rates during steady inlet phases of *SLR*/*FLR* and the influence of total biomass (X_{tot}). (D) Ratios of consumption and loading rates of saccharide and free amino nitrogen. This figure is available in colour online at wileyonlinelibrary.com/journal/brewing.

Table 2. Average composition (±standard deviation) of the green beer (recalculated to 5.2% ethanol) taken from the outflow (Fig. 1l) of the airlift reactor

Beer type	Pilsener Days 0–11	Pilsener Days 11–20	Pilsener Days 20–27	Pilsener Days 27–34	Pilsener Days 34–40	Dark lager Days 40–48	Dark lager Days 48–54
Profile							
Real attenuation (%)	67 ± 3.0	68.8 ± 0.4	67.7 ± 0.47	67.0 ± 1.4	67.3 ± 0.5	66.0 ± 0.0	66.5 ± 0.5
FAN (mg/L)	33.8 ± 4.8	48.2 ± 6.5	60.3 ± 2.8	70.1 ± 2.8	60.0 ± 1.3	50.1 ± 1.8	46.8 ± 0.6
EBU	15 ± 2	15 ± 1	16 ± 0	20 ± 1	20 ± 1	12 ± 0	12 ± 0
Colour (EBC)	17 ± 2	15 ± 0	14 ± 1	18 ± 0	16 ± 0	157 ± 11	159 ± 7
pH	3.8 ± 0.1	4.0 ± 0.0	4.2 ± 0	4.2 ± 0	4.1 ± 0	4.1 ± 0	4.1 ± 0
Volatiles (mg/L)							
Diacetyl	0.44 ± 0.05	0.25 ± 0.03	0.30 ± 0.03	0.31 ± 0.04	0.45 ± 0.03	1.02 ± 0.22	1.20 ± 0.07
Acetaldehyde	17.3 ± 2.8	12.3 ± 2.1	11.5 ± 1.4	11.0 ± 0.3	9.0 ± 0.4	12.5 ± 1.1	11.0 ± 1.1
n-Propanol	37.1 ± 11.4	19.2 ± 0.8	22.4 ± 2.7	26.3 ± 2.9	27.8 ± 1.3	21.4 ± 3.7	17.9 ± 1.4
Isobutanol	23.1 ± 10.1	8.2 ± 1.1	7.9 ± 0.8	9.4 ± 1.0	12.7 ± 0.1	16.7 ± 2.7	18.6 ± 1.9
Amyl alcohols	84.6 ± 24.3	50.5 ± 1.8	53.5 ± 3.5	61.6 ± 5.7	70.5 ± 1.5	78.7 ± 4.6	87.7 ± 5.2
Ethyl acetate	5.2 ± 1.9	11.5 ± 0.9	13.1 ± 0.6	13.9 ± 1.0	20.7 ± 1.0	22.6 ± 1.1	22.1 ± 1.5
Isoamyl acetate	0.20 ± 0.11	0.40 ± 0.02	0.48 ± 0.04	0.70 ± 0.06	1.14 ± 0.05	1.32 ± 0.1	1.56 ± 0.11
A/E	31.2 ± 13.3	6.6 ± 0.8	6.1 ± 0.4	6.6 ± 0.2	5.1 ± 0.1	4.9 ± 0.2	5.3 ± 0.1

EBC, European Brewery Convention; EBU, European Bitterness Units; FAN, Free Amino Nitrogen; A/E, Higher Alcohols to Ester ratio.

nitrogen consumption rate (*FCR*) did not follow *FLR* linearly (Fig. 3D). This could be explained by the uptake rate of amino acids, which takes some time and energy to accomplish *(31)*. Moreover, the initial fall in *FCR*/*FLR* observed in the early stages of continuous fermentation (Fig. 3D) could be ascribed to the increased rates of yeast metabolism and growth, which may have triggered the *de novo* synthesis of amino acids, decreasing the need for external sources of these building blocks. Not surprisingly, *FCR* exerted a strong impact (Fig. 4) on the productivity of higher alcohols (r_p higher alcohols) as the main route for their synthesis is by the catabolism of amino acids through the Ehrlich pathway *(32)*.

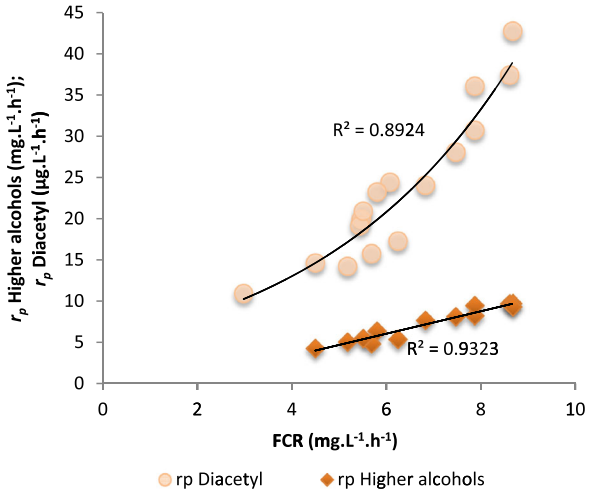

Figure 4. Correlation of free amino nitrogen consumption rate (*FCR*) influencing the productivities of higher alcohols and diacetyl. This figure is available in colour online at wileyonlinelibrary.com/journal/brewing.

Despite the vast presence of precursors as higher alcohols (Table 2), according to some authors, the main factor influencing the synthesis of acetate esters is not substrate availability, but the expression of genes (ATF1, ATF2, Lg-ATF1) encoding alcohol acetyltransferases *(33–35)*. However, the expression of these genes is directly inhibited in the presence of oxygen *(36–40)*. Yet, in order to keep a positive tendency for biomass accumulation, oxygen is crucial for the synthesis of plasmatic membrane structures *(41)*. It is in this context that the core paradox for the feasibility of long-term continuous fermentation emerges: how to build biomass without inhibiting ester synthesis? In batch fermentations, the dissolved oxygen present in wort is rapidly exhausted by the pitched yeast to form new cells. Breweries rely on this initial yeast growth for maturation and crop for re-pitching. Thus, continuous bioreactors as suggested in the current work not only must provide a sensorially balanced green beer, but also need yeast to keep the fermenter working and also to proceed with carbonation and diacetyl removal in the maturation step. Therefore, despite using direct aeration to the airlift reactor, which could impair ester synthesis, the current setup presented consistent acetate ester productivity (Fig. 3).

Fig. 3(C) shows that, in the course of constant inlet of nutrients (stable *SLR* and *FLR*), the growing X_{tot} did not influence consumption and productivity rates. These data suggest that the X_{tot} present during these stages was potentially able to increase the productivities further by raising the *Q*. However, this was not tested owing to logistical difficulties of transporting a higher number of wort barrels from the brewing industry to the laboratory in order to support the increased demand of *SLR* and *FLR*.

Beer analysis

The high initial RT_{tot} needed for building X_{tot} also delayed the washout of the green beer produced during the initial batch phase of the reactor operation. As a result, the green beer from the outflow in the first days of continuous fermentation was sensorially unbalanced, especially for the values of acetaldehyde and higher alcohols (Table 2). Once enough X_{tot} was immobilized inside the airlift reactor and the unbalanced green beer was washed out (~day 10 of the continuous experiment), the higher alcohol to ester ratio (*A/E*) was kept within acceptable values (5.7 ± 0.83) until interruption of fermentation. This ratio was attained both by decreased higher alcohols measured in the green beer after the first days of continuous fermentation and also by an increased rate of ester synthesis when there was sufficient X_{tot} (>30 g/L) in the airlift reactor (Table 2).

The vast majority (~80 %) of higher alcohols present in the final beer are produced by yeast during the primary fermentation either from catabolism *(42,43)* or from the biosynthesis of amino acids *(44)*. These fusel alcohols not only contribute directly to the final beer's aroma and taste, but also contribute indirectly as they are precursors of the most desirable esters. Data involving the production of higher alcohols using ICT is rather inconsistent as some works have reported low production of higher alcohols by immobilized cells *(45–47)*, while others have shown increased biosynthesis when compared with batch fermentations *(48)*. To a large extent, the average total higher alcohols measured in the green beer produced by the current setup were within the expected concentration range *(49,50)* for both Pilsener (119 ± 18 mg/L) and dark lager (142 ± 9 mg/L). In the present work, isoamyl alcohol (3-methyl-1-butanol) and the active amyl alcohol (2-methyl-1-butanol) were measured together and are henceforth referred to as amyl alcohols (Table 2). Bearing in mind that the current work used high-gravity wort (which means that beer produced should still be diluted), not only amyl alcohols, but all other higher alcohols reported in Tables 2 and 3 are in the desired/acceptable concentration range for both Pilsener and dark lager *(49,50)*.

Although esters are lesser constituents of beer, they are extremely important contributors to the aromatic profile of any beer. Just as with the higher alcohols, esters are also mainly produced during the initial vigorous fermentation, which means that a beer's quality depends heavily on a successful primary fermentation. Only acetate esters were measured in the current work because they are present in beer in much higher amounts compared with medium-chain fatty acids ethyl esters. Ethyl acetate is synthesized by the brewing yeast through the action of alcohol acetyltransferase catalysing the condensation reaction between acetyl-CoA with ethanol; it is the most abundant ester in beer. After approximately 10 days of continuous fermentation (X_{tot} > 30 g/L), the average ethyl acetate content in the green beer was 18.4 ± 0.4 and 26.35 ± 0.18 mg/L for Pilsener and dark lager, respectively. These numbers not only agree with other reported setups for primary continuous beer fermentation *(46,51–54)*, but they are also within the desired concentration range *(49,50)*. Isoamyl acetate has a much lower flavour threshold (1.2 mg/L) than its counterpart ethyl acetate (20–30 mg/L) *(49,50)* and it was present below the flavour threshold in the green beer up to the late stages of Pilsener-type wort injection in the current work. It is possible that the increased cell activity driven by increasing *SLR*/*FLR* and biomass itself progressively exhausted the dissolved oxygen, leading to a gradual higher expression of ATF genes *(36)*, and hence higher levels of isoamyl acetate (Fig. 3B).

As reported in the literature involving ICT *(4,6,46,55,56)*, one of the main problems impairing beer produced under such dense pitching rates is the overproduction of diacetyl. This vicinal diketone has a very low odour threshold (around 0.1 mg/L *(50)*), leaving lager beers with an unpleasant sweetish buttery off-flavour. Diacetyl is spontaneously formed outside yeast cells by the non-enzymatic oxidative decarboxylation of α-acetolactate, which in turn is also an intermediate for the *de novo* synthesis of valine. Additionally, the accumulation of α-acetolactate inside

Table 3. Profile of three continuously produced green beers and their corresponding maturated beers. Each shaded row represents a single beer, the number above being the values of green beer and the bold numbers below being the respective final value after maturation

SG (°P)	ABV (%)	Real Att. (%)	pH	Colour (EBC)	EBU	Diacetyl ($mg.L^{-1}$)	Acetaldehyde ($mg.L^{-1}$)	Propanol ($mg.L^{-1}$)	Isobutanol ($mg.L^{-1}$)	Amyl alcohols ($mg.L^{-1}$)	Total Higher Alcohols ($mg.L^{-1}$)	Ethyl acetate ($mg.L^{-1}$)	Isoamyl Acetate ($mg.L^{-1}$)	Total Acetate Esters ($mg.L^{-1}$)	A/E
15.4	6.8	67	4.07	20.8	25	0.64	11.14	34.24	16.21	88.96	139.4	25	1.39	26.45	5.3
15.9	**7.5**	**71.92**	**4.15**	**21.3**	**25**	**0.04**	**8.28**	**35.3**	**16.4**	**90.5**	**142.2**	**36.4**	**2.01**	**38.4**	**3.7**
14.1	6.1	67	4.11	194	14	1.55	12.3	20.7	25.5	104.6	150.8	26.14	1.85	28	5.39
14.8	**6.5**	**67.4**	**4.13**	**213**	**15**	**0.17**	**7.2**	**21**	**19.9**	**99**	**139.9**	**31.9**	**2.31**	**34.2**	**4.1**
14.5	6.3	67	4.10	197	14	1.44	11.53	19	19.6	95.3	133.9	23.7	1.66	25.35	5.28
14.7	**6.5**	**67.39**	**4.12**	**202**	**14**	**0.12**	**6.66**	**23.3**	**17**	**89.4**	**129.7**	**29.9**	**1.88**	**31.8**	**4.1**

SG, Specific Gravity; P, Plato Degree; EBC, European Brewery Convention; EBU, European Bitterness Units; and A/E, Higher Alcohol to Ester ratio.

yeast cells is rate-limiting for the biosynthesis of that amino acid and is therefore excreted *(57)*. As recommended by the EBC, the measurements of total diacetyl performed in the current work also considered the α-acetolactate present in the green beer. Therefore, all values mentioned for diacetyl should be henceforth understood as total diacetyl. As shown in Fig. 4, diacetyl productivity was influenced by *FCR*. Very recently, Krogerus and Gibson *(58)* demonstrated that worts with less FAN produced less diacetyl during fermentation. Before them, Pugh and colleagues *(59)* demonstrated the same correlation, but FAN levels lower than 122 mg/L started to increase diacetyl production. It was clear that depletion of FAN below critical levels stimulated the *de novo* synthesis of valine, increasing the pool of α-acetolactate. Still more recently, Lei *et al. (31)* observed that the intake of valine decreased with increasing FAN content. These authors hypothesized the rise of available FAN to induce yeast to absorb preferred amino acids prior to valine. Valine acts as a feedback inhibitor of ILV2-encrypting acetohydroxy acid synthase, which catalyses the irreversible conversion of pyruvate to α-acetolactate *(60,61)*. Thus, it is possible that the increased *FLR* from day 27 onwards, associated with *FCR* (of preferred amino acids) decreased valine uptake in the current setup, stimulating the synthesis of α-acetolactate, which ultimately led to increased levels of diacetyl (Fig. 4). Regardless of the increased diacetyl productivity, after maturation, the total diacetyl was reduced well below the threshold value for the Pilsener beer (0.04 mg/L) and slightly above for the dark lager (0.12–0.17 mg/L; Table 3).

As maturation time was extended to 30 days, it is possible that some yeast present in maturating bottles had autolysed, slightly increasing the pH in the matured beer (Table 3). Accordingly, the addition of original extract to the bottles certainly changed the quality of matured beer (Table 3). This practice is commonly used to improve the beer's quality in some Belgian ales *(62)*. Yeast metabolism during re-fermentation is completely reductive, owing to the lack of oxygen in the green beer. As a result, yeast will accumulate the reduced coenzymes NADH and NADPH. Therefore, in order to keep the redox balance, the brewing yeast exposed to this situation uses this 'reduction power' to reduce aldehydes to alcohols through the action of aldoketoreductases *(63–65)*. The reduction of aldehydes such as diacetyl and 2-*trans*-nonenal into their respective alcohols improves the beer flavour, as these alcohols have much higher flavour thresholds than the original aldehydes. In the present work, this was true for acetaldehyde, as values were below the threshold *(50)* in the finished beer (Table 3).

Conclusions

The volumetric productivity of the airlift reactor for primary continuous beer fermentation was greatly enhanced by the applied high gravity wort. The current setup has shown not only an extraordinary performance at the level of real attenuation and ethanol productivity, but has also proven to be flexible as two types of beer were produced without interrupting the process.

It should not be forgotten that yeast immobilization was carried out by flocculation, which could bring great economic benefits in further scale-ups of this system. Additionally, this simple self-aggregation method was able to keep an amount of immobilized yeast suspended in the system greater than any other ICT setup reported to date. Moreover, irrespective of the wort type fed into the bioreactor (limits tested in the present work), the concentrations of the main flavour-active compounds

in green beer were in the desired range throughout the entire duration of the continuous experiment. A fine-tuning in the amount of dissolved oxygen provided to the airlift reactor would further improve the quality of the beer produced.

Bearing in mind that this work was a laboratory-based study, faster start-ups of the current setup could be achieved by increasing the initial pitching rate (e.g. using an industrial yeast slurry).

The current work also inspires new studies of diacetyl formation in ICT, which could be triggered by increasing *FLR* and *FCR*. Additionally, it has also been observed that nutrient availability in continuous fermentation can greatly change the manifestations of the physiological state of the yeast population (i.e. viability and fermentation performance rates). Therefore, further work should focus on a deeper understanding of these observations by genetic screening of yeast cells growing and fermenting under such conditions.

Acknowledgements

Eduardo Pires gratefully acknowledges the Fundação para a Ciência e a Tecnologia (FCT, Portugal) for PhD fellowship support (SFRH/BD/61777/2009). All materials supplied by UNICER Bebidas de Portugal, S.A. (S. Mamede de Infesta, Portugal) as well as the technical support of company members Sonia Meireles and Helena Cunha are acknowledged. The financial contributions of the Grant Agency of the Czech Republic (project GAČR P503/12/1424) are also gratefully acknowledged. The authors thank also the Foundation for Science and Technology (FCT-Portugal) for their support through the Strategic Project PEst-OE/EQB/LA0023/2013, also for the PEst-OE/BIA/UI4050/2014; and the Project 'BioInd - Biotechnology and Bioengineering for improved Industrial and Agro-Food processes', ref. NORTE-07-0124-FEDER-000028 Co-funded by the Programa Operacional Regional do Norte (ON.2 – O Novo Norte), QREN, FEDER.

References

1. Verbelen, P. J., De Schutter, D. P., Delvaux, F., Verstrepen, K. J., and Delvaux, F. R. (2006) Immobilized yeast cell systems for continuous fermentation applications, *Biotechnol. Lett. 28*, 1515–1525.
2. Mensour, N. A., Margaritis, A., Briens, C. L., Pilkington, H., and Russell, I. (1997) New developments in the brewing industry using immobilised yeast cell bioreactor systems, *J. Inst. Brew. 103*, 363–370.
3. Willaert, R., and Nedovic, V. (2006) Primary beer fermentation by immobilised yeast – a review on flavour formation and control strategies, *J. Chem. Technol. Biotechnol. 81*, 1353–1367.
4. Brányik, T., Vicente, A. A., Cruz, J. M., and Teixeira, J. A. (2004) Continuous primary fermentation of beer with yeast immobilized on spent grains – the effect of operational conditions, *J. Am. Soc. Brew.Chem. 62*, 29–34.
5. Berlowska, J., Kregiel, D., and Ambroziak, W. (2013) Enhancing adhesion of yeast brewery strains to chamotte carriers through aminosilane surface modification, *World J. Microbiol. Biotechnol. 29*, 1307–1316.
6. Branyik, T., Silva, D. P., Vicente, A. A., Lehnert, R., e Silva, J. B., Dostalek, P., and Teixeira, J. A. (2006) Continuous immobilized yeast reactor system for complete beer fermentation using spent grains and corncobs as carrier materials, *J. Ind. Microbiol. Biotechnol. 33*, 1010–1018.
7. Branyik, T., Vicente, A. A., Cruz, J. M., and Teixeira, J. A. (2002) Continuous primary beer fermentation with brewing yeast immobilized on Spent Grains, *J. Inst. Brew. 108*, 410–415.
8. Tata, M., Bower, P., Bromberg, S., Duncombe, D., Fehring, J., Lau, V. V., Ryder, D., and Stassi, P. (1999) Immobilized yeast bioreactor systems for continuous beer fermentation, *Biotechnol. Progr. 15*, 105–113.
9. Pilkington, H., Margaritis, A., Mensour, N., Sobczak, J., Hancock, I., and Russell, I. (1999) Kappa-carrageenan gel immobilisation of lager brewing yeast, *J. Inst. Brew. 105*, 398–404.
10. Almonacid, S. F., Nájera, A. L., Young, M. E., Simpson, R. J., and Acevedo, C. A. (2012) A Comparative study of stout beer batch fermentation using free and microencapsulated yeasts, *Food Bioprocess Technol. 5*, 750–758.
11. Smogrovicová, D., Dömény, Z., Gemeiner, P., Malovikova, A., and Sturdik, E. (1997) Reactors for continuous primary beer fermentation using immobilised yeast, *Biotechnol. Tech. 11*, 261–264.
12. Dömény, Z., Smogrovicová, D., Gemeiner, P., Sturdík, E., Pátková, J., and Malovíková, A. (1998) Continuous secondary fermentation using immobilised yeast, *Biotechnol. Lett. 20*, 1041–1045.
13. Bester, M. C., Jacobson, D., and Bauer, F. F. (2012) Many *Saccharomyces cerevisiae* cell wall protein encoding genes are coregulated by Mss11, but cellular adhesion phenotypes appear only FLO protein dependent, *G3 (Bethesda) 2*, 131–141.
14. Kurec, M., and Brányik, T. (2011) The role of physicochemical interactions and FLO genes expression in the immobilization of industrially important yeasts by adhesion, *Colloids Surf. B Biointerfaces 84*, 491–497.
15. Stratford, M. (1996) Induction of flocculation in brewing yeasts by change in pH value, *FEMS Microbiol, Lett. 136*, 13–18.
16. Stratford, M., and Carter, A. T. (1993) Yeast flocculation: Lectin synthesis and activation, *Yeast 9*, 371–378.
17. Eddy, A. A. (1955) Flocculation characteristics of yeasts II. Sugars as dispersing agents, *J. Inst. Brew. 61*, 313–317.
18. Powell, C. D., Quain, D. E., and Smart, K. A. (2003) The impact of brewing yeast cell age on fermentation performance, attenuation and flocculation, *FEMS Yeast Res. 3*, 149–157.
19. Panteloglou, A. G., Smart, K. A., and Cook, D. J. (2012) Malt-induced premature yeast flocculation: Current perspectives, *J. Ind. Microbiol. Biotechnol. 39*, 813–822.
20. Chisti, Y., and Moo-Young, M. (1989) On the calculation of shear rate and apparent viscosity in airlift and bubble column bioreactors, *Biotechnol. Bioeng. 34*, 1391–1392.
21. Kaeberlein, M. (2010) Lessons on longevity from budding yeast, *Nature 464*, 513–519.
22. Mortimer, R. K., and Johnston, J. R. (1959) Life span of individual yeast cells, *Nature 183*, 1751–1752.
23. Österlund, T., Nookaew, I., Bordel, S., and Nielsen, J. (2013) Mapping condition-dependent regulation of metabolism in yeast through genome-scale modeling, *BMC Systems Biol. 7*: 36; doi:10.1186/1752-0509-7-36
24. Muller, I., Zimmermann, M., Becker, D., and Flomer, M. (1980) Calendar life span versus budding life span of *Saccharomyces cerevisiae*, *Mechanisms Ageing Devl. 12*, 47–52.
25. Powell, C. D., Van Zandycke, S. M., Quain, D. E., and Smart, K. A. (2000) Replicative ageing and senescence in *Saccharomyces cerevisiae* and the impact on brewing fermentations, *Microbiology 146*, 1023–1034.
26. Deans, K., Pinder, A., Catley, B. J., and Hodgson, J. A. (1997) Effects of cone cropping and serial repitch on the distribution of cell ages in brewery yeast, in *Proc. Eur. Brew. Conv. Congr. City*, pp. 469–476, IRL Press, Oxford.
27. Conrad, M., Schothorst, J., Kankipati, H. N., Van Zeebroeck, G., Rubio-Texeira, M., and Thevelein, J. M. (2014) Nutrient sensing and signaling in the yeast *Saccharomyces cerevisiae*, *FEMS Microbiol. Rev. 38*, 254–299.
28. Gibson, B. R., Lawrence, S. J., Leclaire, J. P., Powell, C. D., and Smart, K. A. (2007) Yeast responses to stresses associated with industrial brewery handling, *FEMS Microbiol. Rev. 31*, 535–569.
29. Puligundla, P., Smogrovicova, D., Obulam, V. S., and Ko, S. (2011) Very high gravity (VHG) ethanolic brewing and fermentation: A research update, *J. Ind. Microbiol. Biotechnol. 38*, 1133–1144.
30. Hasegawa, S., Ogata, T., Tanaka, K., Ando, A., Takagi, H., and Shima, J. (2012) Overexpression of vacuolar H+-ATPase-related genes in bottom-fermenting yeast enhances ethanol tolerance and fermentation rates during high-gravity fermentation, *J. Inst. Brew. 118*, 179–185.
31. Lei, H., Zheng, L., Wang, C., Zhao, H., and Zhao, M. (2013) Effects of worts treated with proteases on the assimilation of free amino acids and fermentation performance of lager yeast, *Int. J. Food Microbiol. 161*, 76–83.
32. Ehrlich, F. (1907) Über die Bedingungen der Fuselölbildung und über ihren Zusammenhang mit dem Eiweissaufbau der Hefe, *Ber. Dtsch. Chem. Ges. 40*, 1027–1047.
33. Zhang, C., Liu, Y., Qi, Y., Zhang, J., Dai, L., Lin, X., and Xiao, D. (2013) Increased esters and decreased higher alcohols production by engineered brewer's yeast strains, *Eur. Food. Res. Technol. 236*, 1009–1014.
34. Saerens, S. M., Verbelen, P. J., Vanbeneden, N., Thevelein, J. M., and Delvaux, F. R. (2008) Monitoring the influence of high-gravity brewing and fermentation temperature on flavour formation by

analysis of gene expression levels in brewing yeast, *Appl. Microbiol. Biotechnol. 80*, 1039–1051.
35. Verstrepen, K. J., Van Laere, S. D., Vanderhaegen, B. M., Derdelinckx, G., Dufour, J. P., Pretorius, I. S., Winderickx, J., Thevelein, J. M., and Delvaux, F. R. (2003) Expression levels of the yeast alcohol acetyltransferase genes ATF1, Lg-ATF1, and ATF2 control the formation of a broad range of volatile esters, *Appl. Environ. Microbiol. 69*, 5228–5237.
36. Fujii, T., Kobayashi, O., Yoshimoto, H., Furukawa, S., and Tamai, Y. (1997) Effect of aeration and unsaturated fatty acids on expression of the *Saccharomyces cerevisiae* alcohol acetyltransferase gene, *Appl. Environ. Microbiol. 63*, 910–915.
37. Malcorps, P., Cheval, J. M., Jamil, S., and Dufour, J.-P. (1991) A new model for the regulation of ester synthesis by alcohol acetyltransferase in *Saccharomyces cerevisiae*, *J. Am. Soc. Brew. Chem. 49*, 47–53.
38. Anderson, R. G., and Kirsop, B. H. (1975) Quantitative aspects of the control by oxygenation of acetate ester formation of worts of high specific gravity, *J. Inst. Brew. 81*, 269–301.
39. Anderson, R. G., and Kirsop, B. H. (1975) Oxygen as a regulator of ester accumulation during the fermentation of worts of high specific gravity, *J. Inst. Brew. 81*, 111–115.
40. Anderson, R. G., and Kirsop, B. H. (1974) The control of volatile ester synthesis during the fermentation of wort of high specific gravity, *J. Inst. Brew. 80*, 48–55.
41. van Meer, G., Voelker, D. R., and Feigenson, G. W. (2008) Membrane lipids: Where they are and how they behave, *Nat. Rev. Mol.Cell Biol. 9*, 112–124.
42. Hazelwood, L. A., Daran, J. M., van Maris, A. J., Pronk, J. T., and Dickinson, J. R. (2008) The Ehrlich pathway for fusel alcohol production: A century of research on *Saccharomyces cerevisiae* metabolism, *Appl. Environ. Microbiol. 74*, 2259–2266.
43. Sentheshanuganathan, S. (1960) The mechanism of the formation of higher alcohols from amino acids by *Saccharomyces cerevisiae, Biochem. J. 74*, 568–576.
44. Oshita, K., Kubota, M., Uchida, M., and Ono, M. (1995) Clarification of the relationship between fusel alcohol formation and amino acid assimilation by brewing yeast using 13C-labeled amino acid, in *Proc. Eur. Brew. Conv. Congr. City*, Brussells, pp. 387–402, IRL Press, Oxford.
45. Ryder, D., and Masschelein, C. (1985) The growth process of brewing yeast and the biotechnological challenge, *J. Am. Soc. Brew. Chem. 43*, 66–75.
46. Smogrovicová, D., and Dömény, Z. (1999) Beer volatile by-product formation at different fermentation temperature using immobilized yeasts, *Process Biochem. 34*, 785–794.
47. Mensour, N., Margaritis, A., Briens, C., Pilkington, H., and Russell, I. (1997) New developments in the brewing industry using immobilised yeast cell bioreactor systems, *J. Inst. Brew. 103*, 363–370.
48. Pajunen, E., Tapani, K., Berg, H., Ranta, B., Bergin, J., Lommi, H., and Viljava, T. (2001) Controlled beer fermentation with continuous on-stage immobilized yeast reactor, in *Proc. Eur. Brew. Conv. Congr. City*, Budapest, chap. 49, pp. 1–12, CD, Fachverlag Hans Carl, Nürenberg.
49. Engan, S. (1981) Beer composition: Volatile substances, in *Brewing Science* (J. R. A. Pollock, Ed.), pp. 93–165, Academic Press, London.
50. Meilgaard, M. C. (1975) Flavour chemistry of beer. Part II: Flavor and threshold of 239 aroma volatiles, *Tech. Q. Master Brew. Assoc. Am. 12*, 151–168.
51. Smogrovicová, D., Dömény, Z., Navrátil, M., and Dvorák, P. (2001) Continuous beer fermentation using polyvinyl alcohol entrapped yeast, in *Proc. Eur. Brew. Conv. Congr. City*, Budapest, chap. 50, pp. 51–59, CD, Fachverlag Hans Carl, Nürenberg.
52. Virkajärvi, I., and Kronlöf, J. (1998) Long-term stability of immobilized yeast columns in primary fermentation, *J. Am. Soc. Brew. Chem. 56*, 70–75.
53. Andersen, K., Bergin, J., Ranta, B., and Viljava, T. (1999) New process for the continuous fermentation of beer, in *Proc. Eur. Brew. Conv. Congr. City*, Cannes, pp. 771–778, IRL Press, Oxford.
54. Kronlöf, J., and Virkajärvi, I. (1999) Primary fermentation with immobilized yeast, in *Proc. Eur. Brew. Conv. Congr. City*, Cannes, pp. 761–770, IRL Press, Oxford.
55. Virkajarvi, I., and Pohjala, N. (2000) Primary fermentation with immobilized yeast: Some effects of carrier materials on the flavour of the beer, *J. Inst. Brew. 106*, 311–318.
56. Kronlöf, J., Härkönen, T., Hartwall, P., Home, S., and Linko, M. (1989) Main fermentation with immobilized yeast, in *Proc. Eur. Brew. Conv. Congr. City*, Zurich, pp. 355–362, IRL Press, Oxford.
57. Dillemans, M., Goossens, E., Goffin, O., and Masschelein, C. (1987) The amplification effect of the ILV5 gene on the production of vicinal diketones in *Saccharomyces cerevisiae*, *J. Am. Soc. Brew. Chem. 45*, 81–84.
58. Krogerus, K. A., and Gibson, B. R. (2013) Diacetyl and its control during brewery fermentation, *J. Inst. Brew. 119*, 86–97.
59. Pugh, T., Maurer, J., and Pringle, A. (1997) The impact of wort nitrogen limitation on yeast fermentation performance and diacetyl, *Tech.Q. Master Brew. Ass. Am. 34*, 185–189.
60. Barton, S., and Slaughter, J. (1992) Amino acids and vicinal diketone concentrations during fermentation, *Tech.Q. Master Brew. Ass. Am. 29*, 60–63.
61. Magee, P. T., and Robichon-Szulmajster, H. (1968) The regulation of isoleucine–valine biosynthesis in *Saccharomyces cerevisiae.* 3. Properties and of the activity of acetohydroxyacid synthetase, *Eur. J. Biochem. 3*, 507–511.
62. Vanderhaegen, B., Neven, H., Coghe, S., Verstrepen, K. J., Derdelinckx, G., and Verachtert, H. (2003) Bioflavoring and beer refermentation, *Appl. Microbiol. Biotechnol. 62*, 140–150.
63. Debourg, A., Laurent, M., Dupire, S., and Masschelein, C. (1993) The specific role and interaction of yeast enzymatic systems in the removal of flavour-potent wort carbonyls during fermentation, in *Proc. Eur. Brew. Conv. Congr. City*, Oslo, pp. 437–444, IRL Press, Oxford.
64. van Iersel, M. F., Eppink, M. H., van Berkel, W. J., Rombouts, F. M., and Abee, T. (1997) Purification and characterization of a novel NADP-dependent branched-chain alcohol dehydrogenase from *Saccharomyces cerevisiae*, *Appl. Environ. Microbiol. 63*, 4079–4082.
65. Van Nedervelde, L., Verlinden, V., Philipp, D., and Debourg, A. (1997) Purification and characterization of yeast 3-methyl butanal reductases involved in the removal of wort carbonyls during fermentation, *Process Biochem. 32*, 447–454.