Research article

Institute of Brewing & Distilling

Received: 21 February 2014 Revised: 18 September 2014 Accepted: 29 October 2014 Published online in Wiley Online Library: 20 January 2015

(wileyonlinelibrary.com) DOI 10.1002/jib.205

Continuous beer fermentation – diacetyl as a villain

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This work tested the viability of producing beer of good quality after maturation of green beer obtained by primary continuous fermentation of high-gravity wort using an airlift bioreactor with flocculated biomass. Fermentation performance of the tested setup was unique as it reached a maximum saccharide consumption rate of 9.43 g L^{-1} h^{-1} and an ethanol productivity of 3.75 g L^{-1} h^{-1}. Despite the high levels of diacetyl present in the green beer, a regular maturation was able to reduce it to below threshold values in up to 15 days. It was observed that diacetyl production was strongly correlated with wort composition injected into the system, rather than with the large amount of biomass immobilized in the bioreactor (up to 727 × 10^6 cell mL^{-1}). Organoleptic tests showed that the maturated beer had no major defects. Copyright © 2015 The Institute of Brewing & Distilling

Keywords: continuous beer fermentation; beer maturation; diacetyl

Introduction

Large breweries are constantly investing in new technologies to increase productivity, with high-gravity brewing (HGB) being the most widespread. However, continuous beer fermentation can be far more productive than HGB. Despite a few disadvantages concerning HGB that have been identified, such as yeast stress affecting cell physiology *(1)* and an unbalanced aromatic profile *(2,3)*, the cost–benefit associated with this technology is certainly favourable. Moreover, for today's large breweries, accelerating processes by using higher fermentation temperatures and yeast selection is an established reality that can provide a final product in <20 days. Nevertheless, continuous fermentation can provide a finished beer in a period as short as 3 days *(4)*. Although continuous fermentation shares some common disadvantages with HGB, the radical change in fermentation process and the associated costs have hindered the decision to proceed with this technology. Thus, to counterbalance the drawbacks, a continuous fermentation setup should offer enough advantages to justify its application.

The first decision to be made concerns the bioreactor type. Airlift reactors are remarkably efficient in mass transfer for either liquid–liquid or solid–liquid phases triggered by rapid mixing, low shear stress, simple design and low energy consumption *(5)*. These qualities have elected airlifts as the reactor of choice for primary continuous beer fermentation *(6–8)*. Another decisive parameter to choose is in regard to the yeast immobilization method. Despite several yeast carriers having been suggested to date *(7–12)*, only a few have been considered for scale-up applications, owing to either their cost or problems affecting the final beer *(4,13)*. The only 'free-of-charge' method of immobilizing yeast in a bioreactor relies on self-aggregation of cells – flocculation.

As continuous fermentations are characterized by a constant supply of amino acids, sugars, oxygen and other nutrients into the bioreactor, the courses of metabolic routes of the yeast cells are naturally changed. For example, the large availability of amino acids can increase higher alcohol production through the Ehrlich pathway *(14)*. Accordingly, the continual supply can trigger a preferential uptake of some amino acids to the detriment of others with lower uptake rates, such as valine or isoleucine *(15,16)*. The affected uptake of valine will ultimately divert metabolism to its synthesis *de novo*, increasing the bulk of the α-acetolactate–diacetyl precursor *(15)*. Also, a deliberate feed of oxygen can directly inhibit the expression of genes encoding crucial alcohol acetyltransferases responsible for catalysing the synthesis of important acetate esters *(17,18)*.

This work evaluates the development of aromatic profiles of beers obtained after maturation of green beer produced by primary continuous fermentation using flocculated yeast in a high-cell-density airlift bioreactor. A key element in the current work was to assess the time required for diacetyl reduction (through regular maturation) in beer produced under continuous operation.

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Material and methods

Brewing yeast

The industrial strain of *Saccharomyces pastorianus* was provided by UNICER Bebidas, S.A. (S. Mamede de Infesta, Portugal). Approximately 1.5 L of yeast slurry was harvested from the storage tank, suspended in 3 L of Pilsner wort (~16 °P), and filled into the airlift reactor.

Wort

An all-grain wort was supplied by UNICER Bebidas, S.A. (S. Mamede de Infesta, Portugal). The wort was collected from the main production line after the cooling stage and prior to aeration. It was filled into pre-autoclaved 20 L barrels (Nalgene, USA) and stored at 2°C during continuous injection to the bioreactor. Two Pilsner wort recipes were used and a detailed composition of each is presented in Table 1.

Continuous fermentation

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 (200 mL min^{-1}) and pressurized air (10 mL min^{-1}). This mixture and flow were kept constant during the course of the experiment. Temperature was held at 15°C using a Julabo F32 Refrigerated/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 employed.

The reactor was sterilized with a 3% (v/v) solution of commercial sodium hypochlorite with 1.5% active chlorine, 48 h prior to use. After this time, the solution was discarded and sterile water was used to wash the reactor. Thereafter, it was filled with a mixture of yeast slurry and 16°P P1 wort as reported above.

Batch growth was kept until complete real attenuation (>67%) was achieved (~24 h). Then the continuous phase started with a total retention time (RT_{tot}) of 23.67 h and a yeast cell count of 3.8 × 10^8 cells mL^{-1}. Beer produced in the first 24 h of continuous fermentation was discarded (i.e. the beer produced in over-batch mode).

Beer maturation

Green beer was stored in a ball lock keg of 20 L capacity. It was placed in the same refrigerator (2°C) as the wort barrel, receiving the green beer through silicon tubing directly from the airlift outflow (Fig. 1). From the keg, green beer was filled into 0.33 L glass bottles with the use of a counter-pressure bottle filler, which ensured that there was little/no oxygen contact with the beer. This process was carried out in two lots of bottles for P1 (P1 and P1′), while P2 was maturated in its own keg. After bottling/locking, the bottles and keg were settled in a cooling chamber (6°C) for maturation.

Table 1. Composition of feeding wort

Recipe	SG (°P)	pH	Colour (EBC)	EBU	FAN (mg L^{-1})
Pilsner 1	15.92	4.93	11.9	28	206
Pilsner 2	13.41	5.07	14.5	29	242

SG, Specific gravity; °P, Plato degree; EBC, European Brewery Convention; EBU, European Bitterness Units; FAN, free amino nitrogen.

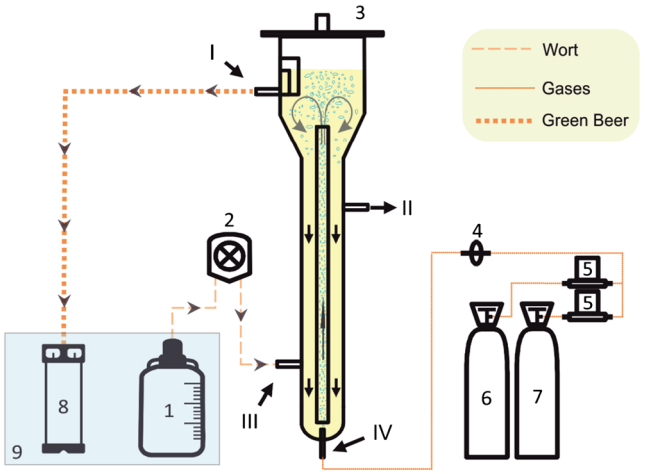

Figure 1. Immobilized yeast bioreactor system used in the current work: 1, wort barrel; 2, peristaltic pump; 3, airlift reactor; 4, air filter; 5, flow mass controllers; 6, pressurized air; 7, CO_2 bottle; 8, ball lock keg as green beer storage; 9, refrigerator at 2°C; I, green beer outflow/sampling point; II, biomass sampling point; III, wort inlet; IV, gas sparger.

Cell count

Yeast immobilized in the airlift reactor was measured daily using an automated cell counter - Z2 (Beckman Coulter, Inc., USA). Samples from the reactor were treated as yeast slurry (owing to the high cell count) and serially diluted following the manufacturer's instructions. Briefly, 1 g of yeast suspension from the airlift reactor was placed into a 100 mL volumetric flask, plus 1 mL of sulphuric acid 2 mol $^{-1}$ (to separate budding yeasts/flocs) and diluted with ISOTON II diluent (Beckman Coulter, Germany). This dilution was followed by two additional dilutions (5:50 and 4:200 mL) before the cell count. Yeast suspended in maturing bottles was directly diluted (400 μL/200 mL) and analysed.

Beer and wort analysis

Characterization of wort, green and maturated beer was performed as recommended in the current Analytica EBC methods. All reagents were purchased from Merck (Germany). Alcohol, specific gravity, original and real extract, pH, colour and degree of fermentation were determined by an automatic beer analyser (Anton Paar Beer Analysis System DMA 4500 GmbH, Austria).

Total diacetyl was determined by gas chromatographic analysis of the static headspace using a Varian CP-3800 gas chromatograph (GC; Varian, USA). Separation was carried using 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 the carrier gas. Samples were automatically injected using a Combi PAL headspace autosampler (CTC Analytics, Switzerland).

Acetaldehyde, higher alcohols and esters were measured using a Varian star 3400 CX GC (Varian, USA) with a flame ionization detector and a thermoconductivity detector. The volatiles

were separated using 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 the carrier. Samples were automatically injected using a Combi PAL headspace autosampler.

The free-amino nitrogen (FAN) and bitterness results were measured in a Varian Spectrophotometer Cary 50 Bio UV–vis (Varian, USA).

Results and discussion

High pitching rates – is it really a threat to beer quality?

As a general rule, all immobilized cell technology (ICT) based systems for continuous fermentation are characterized by a high concentration of yeast in the bioreactor. This abnormal amount of yeast is then ready to continuously process the inlet wort into beer. For example, in the current work, the maximum immobilized biomass obtained through flocculation was 727×10^6 cells mL^{-1}. Flocculation has been avoided as a way of biomass immobilization in ICT systems as it can be affected by many parameters, such as nutrient availability, agitation, calcium levels, pH, temperature, yeast handling and storage conditions *(19–21)*. However, as Table 2 shows, biomass accumulation by flocculation presented an increasing trend throughout the experiment and was unaffected by altering process conditions. This tendency was also observed in a previous experiment *(22)*. Traditional batch fermentations on the other hand use around $10–20 \times 10^6$ cells mL^{-1} as an initial pitching rate, which reaches nearly 70×10^6 cells mL^{-1} after exponential growth. As ICT systems use far greater concentrations of yeast, they often express the biomass concentration in dry weight of yeast per volume. Nonetheless, the estimate (10^9 cells mL^{-1} is equivalent to 50 $g_{dry\ wt}\ L^{-1}$) made by Šmogrovičová *et al. (8)* can be used for comparison. Thus, each 1 $g_{dry\ wt}\ L^{-1}$ would correspond to approximately 20×10^6 cells mL^{-1}. Yeast entrapment methods have been highlighted as the best techniques for attaining extremely high cell loadings *(4)*. These methods normally use between 10^7 and 10^9 cells mL^{-1} of gel matrix *(8,10,23)*. Yet when the beads (biocatalysts) are introduced into the bioreactor, the total immobilized biomass concentration will drop to the same the extent as the solid loading capacity of the system. Tata *et al. (10)* compared a number of two-stage systems for continuous beer production. Each system consisted of two reactors connected in series: two fluidized bed reactors with porous glass beads for cell immobilization; and two loop reactors with a silicon carbide cartridge for yeast load. The maximum loading capacity of yeast immobilization reported by the authors for each of these systems was around 594 and 364×10^6 cells mL^{-1}, respectively. The most promising carrier for cell adhesion was suggested by Brányik *et al. (24)*. The carrier obtained from brewer's spent grains was used in many experiments with promising results *(6,7,25)*. However, despite being simpler and less expensive, the maximum cell loading capacity of systems using brewer's spent grains (~300 x 10^6 cells mL^{-1}) was lower than that of the entrapment-based systems.

The increased productivity rate resulting from greater amounts of yeast either for batch or continuous setups is unquestionable *(6,8,20,26,27)*. Accordingly, the present study obtained uniquely high values of saccharide consumption rate (R_s = 9.43 g L^{-1} h^{-1}) and ethanol productivity (R_p = 3.75 g L^{-1} h^{-1}), higher than any other setup for continuous primary beer fermentation. Additionally, the productivity of the current setup was potentially greater as the real attenuation was always above 70%, even when increasing rates of saccharide loading were used (Table 2). Even under such extreme conditions, the final beer produced had many parameters around the expected values for lager beers and no major defects affecting the aroma profile *(28)*. Increased fermentation rates have been targeted as the result of deficient stress responsiveness by sake yeast strains *(29)*. More recently, it has also been shown that immobilized cells present increased fermentation rates owing to increased expression of RPI1p – a transcription factor that inhibits the Ras/cAMP/PKA pathway *(30)*.

Although increased pitching rates have been identified as detrimental to beer quality *(4,20)*, the demand for higher productivities led to efforts to demystify this effect *(26,27)*. For example, Verbelen *et al. (31)* reported that the negative impact caused by increasing pitching rates in batch fermentations was yeast strain-dependent. Thus it would be possible to take advantage of higher production rates by selecting the correct yeast strain. In a further study, Verbelen *et al. (27)* also concluded that increasing pitching rates only caused minor changes in the beer's flavour-active constituents, with the exception of diacetyl. As in these batch experiments, the only real problem observed in the current work that required closer attention was the diacetyl levels. Yet there is evidence *(22)* that the problem does not necessarily relate to the amount of immobilized yeast, as further discussed.

As the storage keg in this study (Fig. 1) also worked as a sedimentation tank for the excess yeast that came through the outflow, the yeast present in the P1 bottles for maturation was in an

Table 2. Continuous fermentation parameters

Time[a] (h)	RT_{tot} (h)	Yeast cells (10^8 cells mL^{-1})	Real attenuation (%)	SLR	R_s	R_p	FLR	FCR
				($g\ L^{-1}\ h^{-1}$)			($mg\ L^{-1}\ h^{-1}$)	
48	23.67	3.53	70	7.15	5.94	2.36	8.70	6.00
72	19.70	4.04	71	8.59	7.22	2.87	10.46	7.46
96	17.24	5.22	71	9.81	8.64	3.43	11.95	8.24
120	15.10	5.70	70	11.21	9.43	3.75	13.64	9.34
192	15.10	7.27	70	9.35	7.93	3.14	16.03	9.21

Continuous fermentation time including the 24 h washout needed to remove the green beer produced during batch fermentation. RT_{tot}, Total retention time; SLR, saccharide loading rate; R_s, saccharide consumption rate; R_p, ethanol productivity; FLR, free amino nitrogen loading rate; FCR, free amino nitrogen consumption rate.

acceptable amount (~6 × 10^6 cells mL^{-1}). However, as P2 maturation occurred inside its own keg, some of the excess yeast could have started to autolyse, releasing esterases into the maturing beer. This could explain the decrease in isoamyl acetate values observed during maturation (Fig. 2). Horsted *et al. (32)* and Neven *et al. (33)* showed that the excretion of esterases can lead to a considerable change in the ester profile of the beer and the most affected esters are isoamyl acetate, ethyl hexanoate and ethyl octanoate. Taking these facts into account, continuous beer produced in this experiment was of good quality. The beer was blind tasted by a small panel (four people) of professionals at UNICER. The tasting was carried out with the concentrated (not diluted to match 5.2% ethanol) beers shown in Table 3. Continuous P1 beer was considered to be of very good quality and was (overall) considered as similar to the batch-produced beers. However, the diacetyl level of the concentrated P2 was 0.09 mg mL^{-1}, and this could be perceived by all professionals, causing a negative impression. However, after a few days and without the interference of diacetyl, P2 presented a very pleasant (flower-predominant) aroma and a good taste.

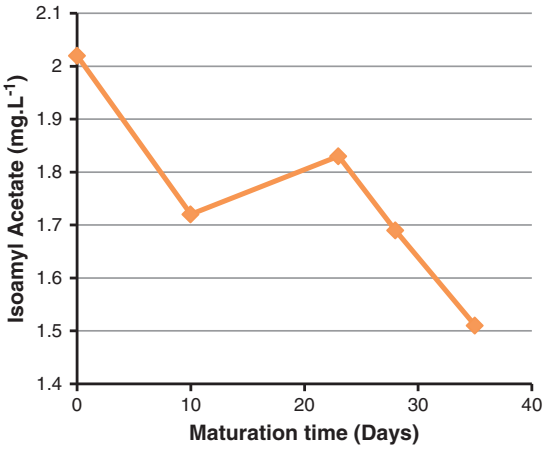

Figure 2. Decrease observed in isoamyl acetate concentrations of Pilsner 2 during maturation inside the keg.

If there is a key parameter affecting continuous beer production, it is the FAN consumption rate (FCR) *(22)*. The environment provided by the current setup (strong agitation, oxygen and high organic loading rate) was very favourable for increased yeast metabolism. This resulted in increased FCR and consequently an increased synthesis of higher alcohols through the Ehrlich pathway *(28)*. As a consequence, the final beer normally had lower FAN levels and increased amounts of fusel alcohols (Table 3). This was evident for P1 but not for P2, which could be explained by the different FAN compositions triggered by the differences between the two recipes. This data also suggests that FCR in continuous fermentation can be controlled by altering the amino acid composition of the fermenting wort. The uptake rate of amino acids is controlled by the presence of preferred amino acids through nitrogen catabolite repression *(34)*. In other words, some amino acids will impair the intake of

Table 3. Main parameters of finished beer (recalculated for 5.2% of ethanol) produced under primary continuous fermentation, compared with batch-fermented beers of the same

Compound (mg L^{-1})	P2 Keg	P2 CC1	P2 CC2	P1	P1′	P1 CC1	P1 CC2
FAN	78.08	83.31	78.94	49.03	43.04	89.18	76.38
Diacetyl	0.07	0.03	0.03	0.03	0.02	0.05	0.04
Acetaldehyde	3.79	2.84	1.85	3.43	3.61	8.70	8.94
n-Propanol	29.47	44.56	33.45	32.01	34.01	21.49	20.31
Isobutanol	14.49	15.04	17.58	20.61	17.60	12.19	14.59
Amyl alcohols	89.42	92.45	92.54	82.90	86.03	75.32	79.60
Total higher alcohols	133.39	152.05	143.57	135.53	137.64	109.00	114.51
Ethyl acetate	27.74	15.77	16.82	13.63	17.15	18.37	20.17
Isoamyl acetate	1.32	1.89	1.89	0.55	0.82	1.48	1.62
Total esters	29.06	17.65	18.71	14.18	17.97	19.86	21.79
A/E	3.58	7.55	6.59	6.60	5.32	3.60	3.35

P2, Pilsner 2; P1, Pilsner 1; P1′, a second lot of P1; CC, cylindro-conical industrial reactors. Shaded columns refer to beer produced by continuous fermentation; A/E, higher alcohols to esters ratio.

others until they are completely exhausted from the wort. Recently, Lei *et al*. *(35)* have shown that FCR increased with the increasing specific gravity of the wort. This is also in agreement with the fact that the brewing yeast needs more assimilable nitrogen to handle intense stress conditions *(36)*. These data also support the increased ratio (0.68) between FCR and FAN loading rate (FLR) experienced during P1 wort (~16°P) inlet, when compared with the FCR/FLR ratio (0.57) of P2 (~13.4°P). On the other hand, yeast entrapment techniques often limit cell proliferation and activity, owing to low mass transfer rates within the matrices. This condition results in a deficient FAN consumption and also imparts an unbalanced flavour profile to the finished beer *(4)*. Recent findings have shown the crucial role of FAN consumption rate in diacetyl formation *(15,16,22)*.

Diacetyl – still a villain!

The main problem affecting beers produced by (either batch or continuous) accelerated fermentations obtained through increased pitching rates is still the diacetyl *(6–8,12,13,15,16)*. Taking the current work as a clear example, the time saved by primary continuous fermentation would not be justified by the 10–20 days required to eliminate the diacetyl (Fig. 3). It is important to remember that commercial breweries already have the technology and know-how to produce a finished product (from the grain to the bottle) in <20 days. While continuous maturation

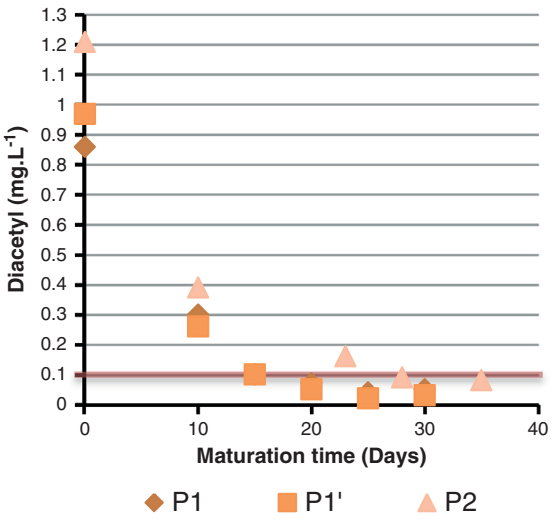

Figure 3. Dynamics of diacetyl reduction from the beers produced under continuous primary fermentation during regular maturation. The line at 0.1 mg L^{-1} marks the threshold of diacetyl for lager beers *(27)*. P1, Pilsner 1; P2, Pilsner 2 P1′, a second lot of P1.

systems have been suggested *(12,37–39)*, the main challenge is to put together both a continuous primary fermentation and a continuous maturation *(40)*. To date, only continuous maturation and alcohol-free beer production are fully implemented on an industrial scale *(4)*. This is because primary fermentation is far more complex and is responsible to a large extent for producing the great majority of the final beer constituents such as ethanol, higher alcohols and esters *(28)*, as well as the synthesis of the diacetyl that must be further reduced through maturation.

Although both vicinal diketones [2,3-butanedione (diacetyl) and 2,3-pentanedione] are formed during fermentations carried out by *Saccharomyces* spp., it is the former that is regarded as the key tracking compound for beer maturation. This is because diacetyl has an approximately 10 times lower flavour threshold than 2,3-pentanedione, and if present in concentrations higher than 0.1 mg L^{-1} *(41)*, confers on lager beers an unpleasant sweetish buttery off-flavour. Accordingly, the current work used diacetyl as a maturation tracker for the beer produced by primary continuous fermentation through regular maturation in bottles or kegs. While P1 beers reached maturation in 15 days after bottling, it took P2 beers 28 days to reach diacetyl values below the threshold within the keg (Fig. 3). The brewing yeast can assimilate and reduce vicinal diketones into diols, which have a much higher flavour threshold. However, diacetyl reduction is not as well understood as diacetyl formation *(16)*. What is known is that, despite the fact that yeast can quickly assimilate and reduce diacetyl, its rate of formation from α-acetolactate is the limiting factor for its reduction *(42)*.

Diacetyl is formed outside the yeast cells by spontaneous non-enzymatic oxidative decarboxylation of α-acetolactate, which in turn is also an intermediate for the *de novo* synthesis of valine. The accumulation of α-acetolactate inside the yeast cell is rate-limiting for the biosynthesis of that amino acid and therefore it is excreted *(43)*. It has been demonstrated that worts with a lower FAN content produce less diacetyl during batch fermentations *(15,16,44)*. However, FAN values <122 mg L^{-1} trigger the opposite response *(44)*. Furthermore, Lei *et al*. *(35)* have shown that the intake of valine decreased with increasing FAN content. Those authors attributed the rise of available FAN to induction in yeast for absorption of preferred amino acids rather than valine. Bearing in mind that valine acts as feedback inhibitor of ILV2-encrypting acetohydroxy acid synthase (which catalyses the irreversible conversion of pyruvate to α-acetolactate), the lack of this amino acid inside the yeast deliberately stimulates α-acetolactate synthesis and hence diacetyl formation *(45,46)*. Therefore, the increased diacetyl in the green beer during the inlet of P2 wort at 192 h of continuous operation of the reactor (Table 4) could be explained by impaired valine intake triggered

Table 4. Profile of green beer sampled from the airlift outflow

Time[a] (h)	SG (°P)	ABV (%)	Real attenuation (%)	pH	Colour (EBC)	EBU	FAN (mg L^{-1})	Diacetyl (mg L^{-1})
48	15.61	7.13	70	3.97	12.9	19	64	1.18
72	15.53	7.22	71	3.81	10.7	14	59	1.23
96	16.26	7.55	71	3.84	10.9	16	64	0.93
120	15.84	7.22	70	3.94	10.4	24	65	1.10
192	13.39	6.04	70	4.19	11.8	21	103	1.70

[a]Continuous fermentation time including the 24 h washout needed to remove the green beer produced during batch fermentation. ABV, alcohol by volume.

by increased availability of preferred amino acids. These pieces of evidence provide valuable insights for future work regarding continuous fermentation with reduced production of diacetyl. Another parameter, which also deserves attention, is in regard to dissolved oxygen, as diacetyl production in ICT can be reduced by lowering the inlet of oxygen into the system *(23)*.

Conclusions

The current work emphasized the feasibility of flocculation as a very promising way of yeast immobilization using an airlift bioreactor. The simple design and good performance offered in the present work may benefit new research on implementing continuous fermentations. It is worth mentioning that flocculation is not only a 'free of charge' immobilization method, but also provides the best conditions for mass/heat transfers as well as for yeast recovery.

This work also suggests that the key parameter affecting the quality of continuously produced beers might be FCR. New insights from recent literature show that the type of amino acids present in wort may be the main factor altering FCR. Thus, the implementation of an appropriate control of FCR in continuous fermentations would provide not only a continuous beer with a balanced aroma profile, but would also solve the main problem – diacetyl. Further research in continuous fermentations should be carried out to confirm this.

Acknowledgements

Eduardo Pires gratefully acknowledges the Fundação para a Ciência e a Tecnologia (FCT, Portugal) for PhD fellowship support (SFRH/BD/61777/2009). All material 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 is gratefully acknowledged. The financial contributions of the EU FP7 project EcoBioCAP – Ecoefficient Biodegradable Composite Advanced Packaging, grant agreement no. 265669 as well as of the Ministry of Education, Youth and Sports of the Czech Republic (MSM 6046137305) are gratefully acknowledged.

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