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ORIGINAL PAPER

Continuous immobilized yeast reactor system for complete beer fermentation using spent grains and corncobs as carrier materials

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Abstract Despite extensive research carried out in the last few decades, continuous beer fermentation has not yet managed to outperform the traditional batch technology. An industrial breakthrough in favour of continuous brewing using immobilized yeast could be expected only on achievement of the following process characteristics: simple design, low investment costs, flexible operation, effective process control and good product quality. The application of cheap carrier materials of by-product origin could significantly lower the investment costs of continuous fermentation systems. This work deals with a complete continuous beer fermentation system consisting of a main fermentation reactor (gas-lift) and a maturation reactor (packed-bed) containing yeast immobilized on spent grains and corncobs, respectively. The suitability of cheap carrier materials for long-term continuous brewing was proved. It was found that by fine tuning of process parameters (residence time, aeration) it was possible to adjust the flavour profile of the final product. Consumers considered the continuously fermented beer to be of a regular quality. Analytical and sensorial profiles of both continuously and batch fermented beers were compared.

Keywords Continuous · Fermentation · Beer · Immobilization · Sensorial analysis

Introduction

During the last decades a broad spectrum of novel inventions revolutionized the whole production chain from barley to beer. Also the traditional beer fermentation and maturation processes using separate open fermentation and closed lager tanks have been often replaced by large production units called cylindroconical tanks. They have proved to be successful both providing operating advantages and ensuring constant quality of the final beer. Conversely, another promising contemporary technology, namely continuous beer fermentation using immobilized brewing yeast, has found only a limited number of industrial applications. The reason for this lies in the often legitimate objections of the industry towards technical difficulties accompanying the process as well as in the desire of the brewers to preserve the traditional image approved by the consumer [9, 23].

However, in such a competitive market as the brewing industry, the potential time saving offered by continuous fermentation present a challenging dilemma to be addressed. Fermentation and maturation are the most time consuming steps in the production of beer, the duration of which is typically between 5–7 and 7–30 days, respectively. The continuous fermentation process based on immobilized yeast cell technology

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allows producing an acceptable end product within as little as 2–3 days [5, 10, 37].

In spite of the economic advantages that continuous beer fermentation offers, difficulties of technical and economic origin have retarded the implementation of the process at industrial scale so far [20, 29, 32]. For example, the total investment costs depend significantly on carrier costs and applied technology. Thus the use of cheap carrier materials in a suitably designed bioreactor could favour the economics of the immobilized process, inspire researchers and encourage brewing engineers.

Brewer's spent grains are a cheap, and food-grade by-product of the brewing industry. They are often used as animal feed [12], rich in fibre and protein, as well as a substrate for mushroom cultivation [39]. Recently, applications of spent grains as a carrier for immobilized cell technologies [1, 9] and raw material for bioethanol production [34] have been studied. Corncobs are large-volume solid wastes that result from sweet corn processing. They are currently either used as animal feed or are returned to the harvested field for land application. Corncobs can also serve as a potential substrate for the production of value-added products (reducing sugars, ethanol, organic acids, etc.) by enzymatic and microbial fermentation processes [13], adsorbents for wastewater treatment [19], microbial carriers in bioremediation [17], additives in thermoplastic polymers [11] and a source of biodegradable plastics [31].

The goal of this work was to study the applicability of these two cellulose-based carrier materials for brewing yeast immobilization in a complete continuous beer fermentation process. The potential carriers were tested in a complete continuous beer fermentation system consisting of a gas-lift and a packed-bed reactor. Attention was paid to optimization of operational conditions (aeration, wort flow rate and process temperature) as well as to long-term process stability. The flavour of the continuously produced beer was evaluated both by methods of instrumental and sensorial analysis and the results discussed in terms of volumetric productivity and sensorial quality of the final product.

Materials and methods

Yeast strain and culture conditions

The brewing (lager) yeast *Saccharomyces carlsbergensis* was supplied by Unicer-Bebidas de Portugal, SGPS, SA (UNICER, SA). The inoculum was cultivated in 500 ml of complex medium under aerobic conditions on a rotary shaker (GFL 3005, Burgwedel, Germany) at 120 rpm, 1 cm stroke, 30°C for 30 h. The composition of the complex medium was (g l^{-1}): KH_2PO_4, 5.0; $(NH_4)_2SO_4$, 2.0; $MgSO_4.7H_2O$, 0.4; yeast extract, 1.0; glucose, 10.0. Medium of the same composition was used during the initial biomass attachment phase. The maturation column (R3) was washed with physiological solution (9 g l^{-1} of NaCl). The all malt wort of original extract 12% (w/w) was supplied by UNICER, SA.

Carrier preparation

Dry spent grains were cleaned by acidic hydrolysis (3 vol% HCl) followed by a delignification in 2% (w/v) NaOH. Prior to use, the carrier was washed several times with water (until neutral pH) and dried [4].

Cylindrical corncobs were cut into slices, diameter ca. 2–3 cm, height ca. 1 cm, and these cylinders were further cut in two pieces along the width. The total volume of the corncob carrier (140 g dry wt) was three times sterilized in distilled water. Between sterilizations it was washed in running water (20 l) in order to remove all flavour active compounds that could interfere with wort/beer.

The immobilized cell reactor system (ICR)

The ICR used in this work (Fig. 1) consisted of a concentric draught tube type gas-lift reactor (GLR) for primary beer fermentation with a total working volume

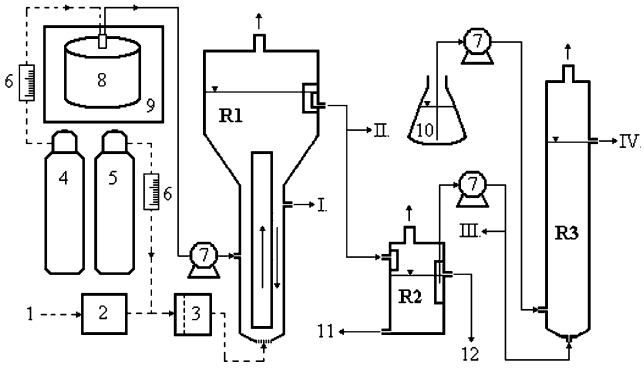

Fig. 1 Immobilized yeast reactor system (*ICR*) for laboratory scale continuous beer fermentation: *1* air supply; *2* mass (gas) flow controller; *3* gas sterilization filter; *4* N_2 bottle; *5* CO_2 bottle; *6* rotameter; *7* peristaltic pump; *8* wort barrel; *9* refrigeration unit; *10* washing (physiological) solution; *11* excess biomass outlet; *12* excess young beer outlet; *R1* main fermentation reactor (*GLR*); *R2* sedimentation tank (*ST*); *R3* maturation reactor (*PBR*); *I* carrier (*spent grains*) sampling point; *II* and *III* young beer sampling points; *IV* maturated beer sampling point

of 2.9 l (R1), a sedimentation tank (ST) for biomass removal with 700 ml volume (R2), and a packed-bed reactor (PBR) for beer maturation with 1.6 l total working volume (R3). The immobilization matrix applied in ICR: R1, spent grain particles; R2, no carrier; R3, corncob cylinders. The dimensions of the concentric draught tube type GLR (R1) with an enlarged top section for degassing were: DC (down comer) height 44 cm, diameter 7 cm; DT (draught tube) height 41 cm, diameter 3.2 cm; CP (cylindrical part) height 8 cm, diameter 14 cm. The angle between the conical sector and the main body was 51°. Gas injection was made through a perforated plate with five holes (diameter 0.5 mm), placed 2.5 cm below the annulus of the riser. The outflow of the reactor was placed behind a sedimentation barrier to minimise carrier losses. Both R2 and R3 were cylindrical reactors with an inside diameter of 8.5 and 7 cm and total working height of 12.5 and 42 cm, respectively. R3 was operated in upward flow. The internal temperatures in R1 (16°C), R2 (9°C) and R3 (2°C) were maintained by means of a cooling coil connected to a refrigeration bath. Air flow into R1 was adjusted using a mass flow controller (Hastings 202D, Teledyne Hastings Instruments, Hampton, Virginia, USA) while the CO_2 flow rate was regulated by a rotameter.

Starting and operating of ICR

The ICR (Plexiglas) was sterilized using sodium hypochlorite solution (2% active chlorine) at least 4 days prior to fermentation. After draining the whole ICR, the sterile gas supply into R1 started (0.4 l min^{-1} of air), and the reactor was filled with 40 g of dry spent grains sterilized in 1.5 l of distilled water. Similarly, the R3 was filled with 140 g of dry corncob particles. Prior to inoculation, the whole ICR was washed with 100 l of sterile water. Subsequently, R1 was charged with complex medium and then inoculated with 1 l of yeast cell suspension. After 24 h of batch growth, the start-up period of R1 was initiated. Since this period was characterised by high medium consumption, (i.e. high total dilution rate D_{tot}), the choice of the complex medium avoided difficulties with supply and storage of wort. In fact, previous experiments showed that the attachment of brewing yeast onto spent grains occurred both in wort and complex medium [5]. The complex medium was initially fed into the ICR at a total residence time (RT_{tot}) of 17 h. The RT_{tot} was decreased to 9 h after 168 h of operation. After 225 h of the start-up period, the complex medium was changed to sterilized wort (50 l, sterilized 40 min at 120°C), which was used throughout the whole fermentation experiment at RT_{tot} = 22–52 h. The wort was kept during the whole experiment in a refrigeration unit (6–8°C) under N_2 atmosphere. During wort fermentation the total gas flow rate (GF, mixture of air and CO_2) into R1 was kept at 0.4 l min^{-1}, with different proportions of air (AF) in the mixture. The ICR was considered to be in steady state after a period of $5 \times RT_{tot}$.

Analytical methods

Characterization of wort, young beer (partially fermented wort leaving R1) and beer (specific gravity, original extract, alcohol, pH, and colour) was performed by an automatic beer analyser (SCABA 5600, Foss Analytical, Slangerupgade, Denmark). Total diacetyl was determined by gas chromatographic analysis of the static headspace [38]. Higher alcohols and esters were measured according to the current EBC recommended methods [2]. The detailed procedure for immobilized biomass (X_{im}) determination can be found in [6]. Cell viability was measured by counting dead cells stained with methylene blue [3].

The single to budding cell ratio was estimated from cell size histograms obtained by Partec Pas III flow cytometer (Partec GmbH, Münster, Germany) equipped with an argon ion laser (488 nm). Typically 10,000 cells were analysed (200–600 cells s^{-1}) and each sample was performed in triplicate.

Samples of free cells were collected at the sampling points of the ICR (Fig. 1). Immobilized cells were liberated from the biocatalyst (carrier + immobilized cells) by agitation. Biocatalyst taken from the ICR was washed in running water (4×100 ml), and then agitated (2 cm magnetic bar, 200 rpm) in 50 ml of physiological solution for 20 min. The biomass released from the carrier was used in flow cytometry and for viability determination.

Sensorial analysis

Consumer acceptance tests (CAT) were carried out by at least 30 untrained consumers of Portuguese nationality with an average profile: 52 ± 6% within age 26–35 years, 73 ± 8% with completed higher education and 80 ± 8% with a weekly consumption of one or more beers. Each CAT was performed on the same day in a controlled room (temperature, noise, individuality of the taster) so that unbiased results were obtained. Samples of both continuous and commercial beer brands were poured without foam into a black glass (90 ml) and tasted at temperatures between 6 and 8°C. Results were analyzed by Analysis of Variance (ANOVA) and Tukey tests ($P \leq 0.05$), to quantify the

variability in the average of responses. The samples of continuous beer for both CAT and descriptive evaluation tests (DST) were collected under N_2 atmosphere 24 h before tasting and stored in PET bottles at 4°C.

Seven tasters of the internal sensory panel of UNICER, SA with at least 1 year of sensory experience were recruited based on their good sensory ability. The DST took place once per week in the morning hours in an adequately isolated taste room. Panellists were asked to describe the flavour profile according to description test. The samples were tasted at 12°C and evaluated using a nine point scale (0 = absent, 1–3 = low, 4–6 = moderate, 7–9 = strong). An average of each sensorial attribute was calculated to evaluate the flavour profile of samples.

Results

Extract consumption

According to the results (Fig. 2), about 79.5 ± 7.1% of the total fermentable extract is consumed in the gas-lift reactor (R1). The contribution of the sedimentation tank (R2) and maturation column (R3) to the fermentation in the ICR were on average 10.8±4.7 and 9.3±2.8%, respectively. The contribution of R2 is not excessive thanks to the short residence time in R2 (RT_{R2} = 3–7 h). At RT_{tot} > 43 h the beer extract was almost independent on RT_{tot} and close to limit achievable for the supplied wort (Fig. 2). This limit is determined by the portion of fermentable sugars (ca. 70%) in the original extract of wort (12% w/w).

The influence of aeration in R1 on wort fermentation was less noticeable (data not shown). Although the nitrogen sparging into the wort barrel decreased the wort dissolved oxygen to ca. 1 mg l^{-1}, a significant amount of oxygen entered the feed stream through silicon tubing (2.5 m long, 3 mm in diameter). The dissolved oxygen concentration entering into R1 with wort was from 5 to 7 mg l^{-1} due to flow rates ranging from 260 to 100 ml h^{-1}, respectively.

Biomass growth and cell viability

In the course of the continuous experiment the dead immobilized cell number in R1 increased from 7.5 to 17% of total immobilized cells (Fig. 3). The immobilized biomass concentration on spent grain particles in R1 ($X_{im(R1)}$) reached its plateau after ca. 4 days of feeding with wort and then it remained rather constant ($X_{im(R1)}$ = 0.63±0.023 g_{IB} g_C^{-1}; IB, immobilized biomass; C, dry carrier). The average concentration of free cells in the outflow from R1 was 3.9±1 g l^{-1} and it varied in accordance with growth conditions.

Due to the regular removal of excess biomass, the dead cell number in the outflow from R2 did not exceed 8% of total cells in the outflow (Fig. 3). The main reason for the inclusion of R2 into the ICR was to remove free biomass from the product stream before entering R3. However, the effectiveness of biomass removal was only 44±11%. As a consequence of this, the concentration of cells at the entrance to R3 was 2.2±0.8 g l^{-1}. Since the average cell content in the outflow from R3 was only 1.3±0.5 g l^{-1}, it was necessary to remove the excess of gradually accumulating biomass in R3. Such removal was achieved by a

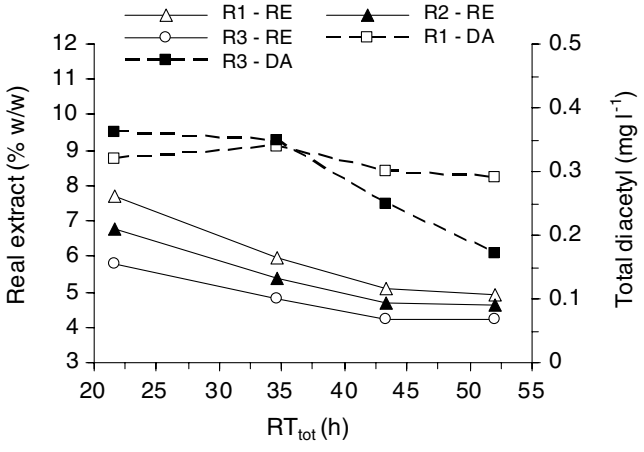

Fig. 2 The influence of total residence times (RT_{tot}) on total diacetyl formation (*DA*) and original extract (12% w/w) consumption, shown as real extract (*RE*) remaining in each stage of the ICR: *R1* main fermentation reactor (16°C), *R2* sedimentation tank (9°C), *R3* maturation column (2°C). Air flow (AF = 0.02 l min^{-1}) and total gas flow rate (GF = 0.4 l min^{-1}) into R1 were constant

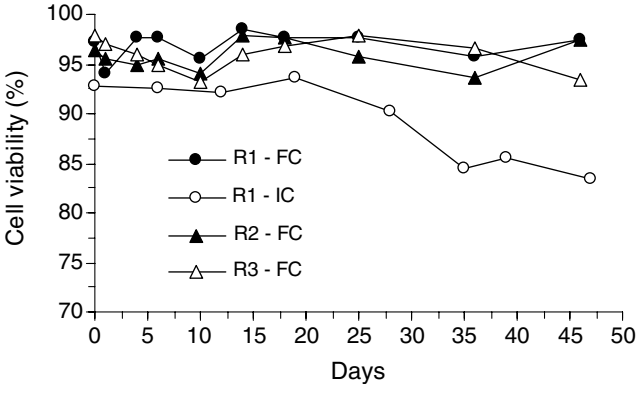

Fig. 3 Percentage of viable cells among free cells (*FC*) in each reactor of the ICR (*R1*, *R2* and *R3*) and among immobilized cells (*IC*) in R1

periodical (every 10 days) upward flushing of R3 with 4 l of sterile physiological solution.

The viability of cells in the outflow from R3 was between 93 and 98% (Fig. 3). Because of difficulties with sterile carrier sampling from R3 it was not possible to determine immobilized cell biomass ($X_{im(R3)}$) and viability in the course of beer fermentation. However, after dismantling ICR the $X_{im(R3)}$ distribution on corncob particles was 0.354, 0.56 and 0.61 $g_{IB}\,g_C^{-1}$ at the top, middle and bottom of the R3, respectively. The corresponding immobilized cell viabilities in R3 were 93, 86.3 and 75%.

The budding to single cell ratio of a yeast population reflects the intensity of growth in a particular system. The average percentage of budding cells in each individual stage of the ICR during the whole fermentation experiment was decreasing in the following order: free cells in R1 (25.2 ± 5.9%) > free cells in R2 (21.5 ± 3.0%) > free cells in R3 (18.4 ± 3.6%) > immobilized cells in R1 (15.6 ± 0.9%) > immobilized cells in R3 (15.2 ± 1.3%). The average deviations of the mean values show that the free cells responded to changing aeration and feed rate by altered growth intensity, while the percentage of single immobilized cells remained rather constant.

Analytical parameters of continuous beer

Comparison of the selected analytical parameters of continuously produced with industrially produced lager beer revealed a satisfactory similarity (Table 1), except for diacetyl (Fig. 2) and higher alcohols to esters ratio (Table 1).

At constant aeration into R1 (20 ml min^{-1}), the total diacetyl (diacetyl + α-acetolactate) level in R1 was relatively stable (0.35–0.29 mg l^{-1}) and almost independent on RT_{tot} (Fig. 2). At short residence times (RT_{tot} = 22–35 h), the continuing wort fermentation in R2 and R3 resulted in further increase of diacetyl concentration in these stages of the fermentation (Fig. 2). Conversely, at long RT_{tot} the fermentable extract of the wort entering into R3 was almost fully consumed allowing thus a partial re-assimilation of diacetyl in R3 (Fig. 2). Although the total diacetyl in the outflow from R3 decreased with increasing RT_{tot} in ICR (Fig. 2), at RT_{tot} = 52 h it still slightly exceeded the taste threshold (0.15 mg l^{-1}) for lager beers [22]. With increasing aeration, the diacetyl formation in R1 grew more intensive (Table 1).

Higher alcohols (fusel alcohols), and esters in particular, contribute significantly to the quality of finished

Table 1 Process and analytical parameters of beer produced in the ICR compared with a control beer produced industrially by traditional technology, and the flavour thresholds of major volatiles in lager beers

Parameter	CB1[a]	CB2[b]	CB3[c]	CB4[d]	Control[e]	Threshold [22]
Total residence time (h)	29	43	52	52	–	–
Aeration into R1 (ml min^{-1})	0	10	20	50	–	–
Original extract (% w/w)	12	12	12.4	12	12.1–12.5	–
Alcohol (v/v%)	4.89	5.3	5.51	5.04	5.6	–
Real extract (% w/w)	4.7	3.72	3.9	3.84	4.1–3.7	–
PH	4.4	4.23	4.33	4.36	4.0–4.4	–
Bitterness (EBU)	21	25	21	–	20–24	–
Total diacetyl in R1 (mg l^{-1})	0.24	0.25	0.29	0.32	–	–
Total diacetyl (mg l^{-1})	0.29	0.25	0.17	0.25	0.03	0.15
n-Propanol (mg l^{-1})	16.1	21.7	32.3	28.7	17.5 [7]	800
Isobutanol (mg l^{-1})	8.3	10	12	10	12.5 [7]	200
Amyl alcohols (mg l^{-1})	55.8	63.6	71.1	60.3	70 [7]	70
Total higher alcohols (mg l^{-1})	80.2	95.3	115.3	99	100 [7]	–
Ethyl acetate (mg l^{-1})	11.3	5.1	4.2	3.54	17.2 [7]	30
Amyl acetates (mg l^{-1})	0.2	0.2	0.1	0.03	1.2 [7]	1.2
Total esters (mg l^{-1})	11.5	5.3	4.3	3.6	18.4 [7]	–
A/*E*[f]	7	18	27	27.5	5–6 [7]	–
Acetaldehyde (mg l^{-1})	13.1	20.2	26	36	ca. 8	10

R1 main fermentation reactor; If not specified, the parameter values correspond to finished beer

[a]Continuous Beer 1

[b]Continuous Beer 2

[c]Continuous Beer 3

[d]Continuous Beer 4

[e]Industrially produced, unfiltered and non-pasteurized Portuguese lager 1

[f]Higher alcohols to esters ratio

beer. The higher alcohols are yeast growth related flavour compounds, which are produced during sugar and wort amino acid assimilation [18, 27]. Their total concentration at the outflow from the ICR was similar to control beer (Table 1). The influence of oxygen supply into R1 on ester formation was unambiguous. The final concentration of esters was significantly lower comparing to control beer and decreased with aeration (Table 1). Therefore the higher alcohols to esters ratio (*A/E*) in continuously produced beers was spread in a wide range from comparable to significantly higher values than those in control beer (Table 1). The acetaldehyde content in the continuously fermented beers was higher than in control beer and it increased with aeration into R1 (Table 1).

Descriptive sensorial tests (DST)

Experienced tasters carried out three DST classifying in each the taste and aroma profile of one unfiltered and non-pasteurized beer produced in ICR at different process conditions and an unfiltered and non-pasteurized commercial Portuguese lager (control beer). The intensities of flavour features of the examined beer were estimated on a nine point scale (0 = absent, 1–3 = low, 4–6 = moderate, 7–9 = strong).

The results of DST revealed the most significant difference between the compared beers in higher fruity (estery) fragrance and intensive bitterness of continuously fermented beers (Fig. 4).

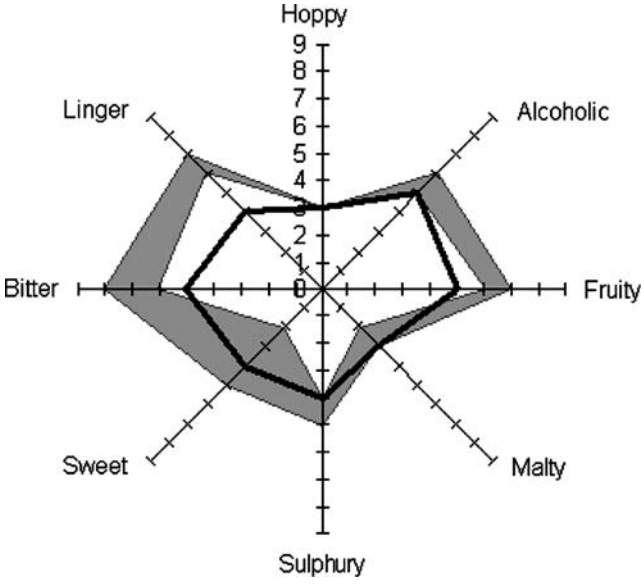

Fig. 4 Comparison of the flavour/aroma features of industrial control beer (*thick line*) with beer produced in the ICR (*grey area*) during DST

Consumer acceptance tests (CAT)

In order to find out the acceptance of continuously fermented beers by consumers a group of at least 30 untrained tasters carried out three CAT, classifying in each of them three different lager beers on a nine point scale (1, the worst conceivable; 9, the best imaginable). The results of the comparison of the unfiltered and non-pasteurized beer from ICR with various bottled commercial lager beers are shown in Table 2. The CAT 1 and CAT 2 revealed a consumer preference in favour of Portuguese lager beers manifested by a statistically significant difference. The average classification of beers in CAT 3 did not show any statistically significant difference (Table 2).

Discussion

The percentage of budding cells among total immobilized biomass in R1 was almost constant and independent on growth conditions (15.6 ± 0.9%). Thus, the fraction of actively growing immobilized cells in R1 ($X_{im(act)} = X_{im(R1)} \times 0.156 = 0.63 \times 0.156 \approx 0.1\ g_{IB}\ g_C^{-1}$) was found to be close to previously published results [8]. This is supporting the existence of growing yeast biofilm fraction, most probably the top layer of yeast biofilm, capable of self-regeneration based on biofilm detachment and subsequent re-colonization of carrier [8].

High dead cell numbers have been observed in continuous main fermentation systems such as 17% of total cells in R1 after 47 days at 16°C, 20% in packed-bed column with DEAE-cellulose after 56 days at 10°C [14] and 24% in gas lift with κ-carrageenan after 60 days at 12–22°C [30]. This may already have practical consequences such as altered fermentation rate

Table 2 Average classification of commercial lagers and beers from the ICR during consumer acceptance tests (CAT)

	CB1[a]	CB5[b]	PL1[c]	PL2[d]	SL[e]	AL[f]	CL[g]
CAT 1		6.1^{SD}	7.0^{SE}	6.8^{SE}			
CAT 2		5.8^{SE}	6.8^{SD}		6.0^{SE}		
CAT 3	5.9^{SE}					6.4^{SE}	6.0^{SE}

SE Statistically equal; *SD* Statistically different

[a]Continuous Beer 1 (no aeration in R1, RT_{tot} = 29 h)

[b]Continuous Beer 5 (AF in R1 = 20 ml min^{-1}, RT_{tot} = 35 h)

[c]Portuguese lager 1

[d]Portuguese lager 2

[e]Spanish lager

[f]American lager

[g]Czech lager

and by-product formation, cell autolysis, etc. Consequently, when dead immobilized cell numbers significantly exceed the values acceptable in batch fermentations (ca. 5%), the need for a biocatalyst replacement is relevant [15]. In an agitated gas-lift reactor the ''old'' biocatalyst slurry can be easily replaced by clean spent grains [8], while in packed-bed columns with corncob particles a turbulent washing can be applied.

In this work a regular washing of R3 was required because of gradual biomass accumulation. In the future this could be improved by construction innovations of R2, such as increased height to diameter ratio (H/D > 1.5) and conical shaped bottom, leading to improved biomass removal. Of course, any other yeast removal technique (e.g. continuous centrifugation) can be applied in industrial scale as well [24].

The effect of operational conditions on the formation of organoleptically active compounds during continuous beer fermentation is crucial and has already been intensely studied [7, 25, 33]. Oxygen supply has been reported to be critical for balanced beer flavour since, e.g. excess aeration leads to biomass over-production, low ester but strong acetaldehyde, diacetyl and fusel alcohol formation [7, 26].

Two processes contribute to the oxygen supply to R1: (1) the gas–liquid oxygen transfer in R1 (GLR) and (2) the dissolved oxygen entering with wort feed. The oxygen transfer from the gas phase into the reactor liquid can be estimated taking into account the oxygen solubility in water ($K_{O_2} = 47.5 mgO_2 l^1$ at 16°C), the partial oxygen pressure in the driving gas (p_{O_2} = 0.00525–0.02625 for AF = 10–50 ml min^{-1} in GF = 400 ml min^{-1}), the oxygen mass transfer coefficient k_La = 8.45 h^{-1} in a similar gas-lift reactor [36], and by assuming that the steady state dissolved oxygen concentration during beer fermentation was zero. Although both K_{O_2} and k_La may be overestimated, since they were determined for clear water and a two-phase system without solids, the oxygen load values (6.1–30.5 mgO_2 h^{-1}) for direct aeration of R1 are significantly higher than those of the wort oxygen load. The oxygen load entering into R1 varied with wort flow rate (0.7–1.3 mgO_2 h^{-1}).

The formation of diacetyl from α-acetolactate is linked through the valine anabolic pathway with biomass growth during wort fermentation [28]. For this reason, the majority of the diacetyl formation in the ICR occurred in R1 (Fig. 2). Oxygen, as a growth stimulator, also increased diacetyl formation in R1 (Table 1).

Diacetyl is enzymatically removed from young beer by brewing yeast via reduction to acetoin and further to 2,3-butanediol. This process depends on temperature, yeast concentration and on factors which promote or prolong an extensive contact between yeast and diacetyl in young beer [40]. We assume that in order to decrease the diacetyl concentration in beer produced by ICR below its taste threshold, an increased residence time in the maturation column (RT_{R3}) would be recommended rather than an increase of RT_{tot}. This could be achieved by enlarging the existing R3 in proportion to R1. Obviously, other strategies of enhanced maturation, e.g. heat treatment of young beer, should be pondered too [41].

Besides diacetyl there are other by-products of fermentation, namely higher alcohols and esters, having considerable sensorial effects on beer. It is known that the excess of aeration inhibits the cytosolic alcohol acetyltransferase, responsible for ester formation in yeast [21]. Consequently, the sudden decrease in ester production (Table 1) and a higher than optimum fusel alcohol to esters (A/E) ratio in continuous beers can be explained by the excess oxygen supply through direct aeration of R1 (Table 1).

Acetaldehyde is a normal intermediate of alcoholic fermentation. Its level in lager beers usually varies in the range 2–20 mg l^{-1} [35]. The acetaldehyde content in continuously fermented beers was higher than in control, and increased with air supply into R1 (Table 1) [16].

Some substances in beer can be measured specifically; however, there are taste and aroma attributes in beer which can not be determined analytically. Many of them make the beer attractive to the consumers. The major differences between continuous and industrial batch beers detected by trained tasters were in bitterness together with its lingering character and to a smaller extent in its fruity taste (Fig. 4).

Controversially, the intensity of the bitterness detected by experienced tasters was significantly higher than the bitterness units (EBU—bitter substances in beer, mg l^{-1}) when comparing both parameters to control beer (Fig. 4). It can be hypothesized, that this difference is due to excessive presence of cold break polyphenols (tannins) in continuous beers. The cold break that appears in cold wort consists of small particles (ca. 0.5 μm) made of protein–polyphenol–carbohydrate compounds. The higher cold break content (above 200 mg l^{-1}) may change the perception of bitterness intensity and character (lingering) by tasters [15]. In traditional lager beer production the cold break is removed either with collapsed foam and/or it is retained by flocculating brewing yeast. However, in ICR, neither foam removal nor sufficient cell sedimentation occurs. To avoid undesirable bitterness, a reduced hop

addition during wort boiling or a partial removal of cold break from unfermented wort by filtration, centrifugation or flotation (air bubbles) should be considered.

Although the formation of esters, which cause unpleasant fruity tastes at higher concentrations, was suppressed in the ICR by excessive oxygen supply (Table 1), the tasters reported a ''fruitlike'' aftertaste in continuous beers (Fig. 4). The higher acetaldehyde content (Table 1) may contribute to this fruitiness of continuous beers since it causes ''green'' young flavours or musty aromas at concentrations above 20 mg l^{-1} [15, 35]. Furthermore, amyl alcohols may cause fruity flavours at threshold concentrations of ca. 70 and 65 mg l^{-1} for 2- and 3-methylbutanol, respectively [22]. However, the concentration of amyl alcohols found in continuous beers was similar to that of the industrial product (Table 1).

The sweetness of the continuous beers varied, in the opinion of the panellists, in a rather wide range (Fig. 4). This feature of the final product was possible to control most effectively through residence time. Nevertheless, the final taste profile results from interplay of different taste features, e.g. the sweetness was apparently influenced the bitterness. Namely, the sweeter the beer was found, the lower intensity of bitterness was attributed to it (data not shown).

When evaluating product quality, one has to bear in mind that the expected beer flavour is influenced by its type and circumstances depending on country and fashion. Thus, it is essential to assess the acceptability of a new product to the consumer. Moreover, beer is a drink the taste of which easily becomes a familiar one and this information stays registered in the memory. This can explain the highest average classification of the local beer brands (PL 1 and 2) during CAT (Table 2). Taking this into account, the absence of statistically significant difference between continuous beers and the compared foreign beer brands means, that the local consumers considered the taste of the continuous beers fully acceptable, although slightly distinctive from the Portuguese-made products.

Conclusions

In order to convince the brewing engineers and economists that continuous brewing can produce both quality and savings, the researchers should not lose sight of the applicability, simplicity and economic attractiveness of the suggested fermentation systems. Accordingly, the investment costs (e.g. carrier price) of the continuous beer fermentation should be kept as low as possible. The results above show that the continuous reactor system (ICR) containing brewer's yeast immobilized on cheap carriers (spent grains, corncobs) was able to operate steadily for almost 2 months.

The finished product from ICR was found by consumers to be quite acceptable from a flavour perspective, although a little distinct from the local lager beer brands produced by conventional technology. The descriptive sensorial analysis carried out by experienced tasters also found differences between the products of continuous and batch fermentation, though some of them can be eliminated by process optimization. For instance (1) the diacetyl content can be decreased by a prolonged maturation in an enlarged maturation column; (2) the prevention of over-aeration ensures an optimum formation of volatile compounds; (3) the palatefulness (body) of the final beer can be controlled through the residence time determining the wort extract consumption; (4) an ideal bitterness would require adjusted hop addition and/or cold break removal from wort.

Generally, achieving the desired flavour balance of beer is a challenging task, but the process parameters provide an effective tool of its adjustment. Moreover, some ''faults'' of the continuous beer found by trained tasters would be eliminated in large scale operation, since they arose from the necessity (in this particular work) of additional wort sterilization (leading to oxidized or burnt aftertaste). The presented continuous fermentation system stands out by its significantly shorter fermentation times, cheap carrier materials reducing the investment costs and beer with generally acceptable and sufficiently balanced flavour.

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References

1. Almeida C, Brányik T, Moradas-Ferreira P, Teixeira JA (2003) Continuous production of pectinase by immobilized yeast cell on spent grains. J Biosci Bioeng 96:513–518
2. Analytica-EBC (2000) European brewery convention. Fachverlag Hans Carl, Nürnberg
3. Analytica-Microbiologica-EBC (2001) European brewery convention. Fachverlag Hans Carl, Nürnberg, Section 3, Method 3.2.1.1, Germany
4. Brányik T, Vicente AA, Machado Cruz JM, Teixeira JA (2001) Spent grains—a new support for brewing yeast immobilization. Biotechnol Lett 23:1073–1078
5. Brányik T, Vicente AA, Machado Cruz JM, Teixeira JA (2002) Continuous primary beer fermentation with brewing yeast immobilized on spent grains. J Inst Brew 108:410–415

6. Brányik T, Vicente A, Oliveira R, Teixeira JA (2004) Physicochemical surface properties of brewing yeast influencing their immobilization to spent grains in a continuous reactor. Biotechnol Bioeng 88:84–94
7. Brányik T, Vicente AA, Machado Cruz JM, Teixeira JA (2004) Continuous primary fermentation of beer with yeast immobilized on spent grains—the effect of operational conditions. J Am Soc Brew Chem 62(1):29–34
8. Brányik T, Vicente AA, Kuncová G, Podrazký O, Dostálek P, Teixeira JA (2004) Growth model and metabolic activity of brewing yeast biofilm on the surface of spent grains: a biocatalyst for continuous beer fermentation. Biotechnol Progr 20:1733–1741
9. Brányik T, Vicente AA, Dostálek P, Teixeira JA (2005) Continuous beer fermentation using immobilized yeast cell bioreactor systems. Biotechnol Progr 21:653–663
10. Dömény Z, Šmogrovičová D, Gemeiner P, Šturdík E, Pátková J, Malovíková A (1998) Continuous secondary fermentation using immobilised yeast. Biotechnol Lett 20(11):1041–1045
11. Georgopoulos ST, Tarantili PA, Avgerinos E, Andreopoulos AG, Koukios EG (2005) Thermoplastic polymers reinforced with fibrous agricultural residues. Polym Degrad Stabil 90(2):303–312
12. Gondwe TNP, Mtimuni JP, Safalaoh ACL (1999) Evaluation of brewery by-products replacing vitamin premix in broiler finisher diets. Indian J Anim Sci 69(5):347–349
13. Hang YD, Woodams EE (2001) Enzymatic enhancement of citric acid production by *Aspergillus niger* from corncobs. Lebensm-Wiss Technol 34(7):484–486
14. Kronlöf J, Härkönen T, Hartwall P, Home S, Linko M (1989) Main fermentation with immobilized yeast. In: Proceedings of the European Brewing Convention Congress. Zurich IRL Press, Oxford, pp 355–362
15. Kunze W, Mieth HO (1996) Technology brewing and malting, Chapter 11. VLB, Berlin
16. Kuriyama H, Kobayashi H (1993) Effect of oxygen supply on yeast growth and metabolism in continuous fermentation. J Ferment Bioeng 75:364–367
17. Labana S, Pandey G, Paul D, Sharma NK, Basu A, Jain RK (2005) Pot and field studies on bioremediation of p-nitrophenol contaminated soil using *Arthrobacter protophormiae* RKJ100. Environ Sci Technol 39(9):3330–3337
18. Landaud S, Latrille E, Corrieu G (2001) Top pressure and temperature control the fusel alcohol/ester ratio through yeast growth in beer fermentation. J Inst Brew 107:107–117
19. Leyva-Ramos R, Bernal-Jacome LA, Acosta-Rodriguez I (2005) Adsorption of cadmium (II) from aqueous solution on natural and oxidized corncob. Sep Purif Technol 45(1):41–49
20. Linko M, Haikara A, Ritala A, Penttilä M (1998) Recent advances in the malting and brewing industry. J Biotechnol 65:85–98
21. Masschelein CA (1997) A realistic view on the role of research in the brewing industry today. J Inst Brew 103:103–113
22. Meilgaard MC (1975) Flavor chemistry of beer. II. Flavor and threshold of 239 aroma volatiles. Technical Quarterly, Master Brewers Association of Americas 12:151
23. Mensour NA, Margaritis A, Briens CL, Pilkington H, Russell I (1997) New developments in the brewing industry using immobilised yeast cell bioreactor systems. J Inst Brew 103:363–370
24. Nakanishi K, Murayama H, Nagara A, Mitsui S (1993) Beer brewing using an immobilized yeast bioreactor system. Bioprocess Technol 16:275–89
25. Norton S, D'Amore T (1994) Physiological effects of yeast immobilization: application for brewing. Enzym Microb Tech 16:365–375
26. Okabe M, Katoh M, Furugoori F, Yoshida M, Mitsui S (1992) Growth and fermentation characteristics of bottom brewer's yeast under mechanical stirring. J Ferment Bioeng 73:148–152
27. Pajunen E (1996) The behaviour of immobilized yeast cells. Cerevisia 4:33–37
28. Petersen EE, Margaritis A, Steward RJ, Pilkington PH, Mensour NA (2004) The effect of wort valine concentration on the total diacetyl profile and levels late in batch fermentations with brewing yeast *Saccharomyces carlsbergensis*. J Am Soc Brew Chem 62:131–139
29. Pilkington PH, Margaritis A, Mensour NA, Russel I (1998) Fundamentals of immobilized yeast cells for continuous beer fermentation: a review. J Inst Brew 104:19–31
30. Pilkington H, Margaritis A, Mensour N, Sobczak J, Hancock I, Russel I (1999) Kappa-Carrageenan gel immobilization of lager brewing yeast. J Inst Brew 105:398–404
31. Schilling CH, Tomasik P, Karpovich DS, Hart B, Garcha J, Boettcher PT (2005) Preliminary studies on converting agricultural waste into biodegradable plastics. Part II: Corncobs. J Polym Environ 13(1):57–63
32. Shen H-Y, Moonjai N, Verstrepen KJ, Delvaux FR (2003) Impact of attachment on yeast physiology and fermentation performance. J Am Soc Brew Chem 61:79–87
33. Shen H-Y, De Schrijver S, Moonjai N, Verstrepen KJ, Delvaux F, Delvaux FR (2004) Effects of CO_2 on the formation of flavour volatiles during fermentation with immobilized brewer's yeast. Appl Microbiol Biot 64:636–643
34. Shindo S, Tachibana T (2005) Production of bioethanol from spent grain, a by-product of beer production. J Biotechnol 118:S165–S166
35. Šmogrovičová D, Dömény Z (1999) Beer volatile by-product formation at different fermentation temperature using immobilized yeast. Process Biochem 34:785–794
36. Sousa ML, Mota M, Teixeira JA (1994). Influence of operational parameters on the start-up of a flocculation airlift bioreactor. Colloids Surf B Biointerfaces 2:181–188
37. Tata M, Bower P, Bromberg S, Duncombe D, Fehring J, Lau V, Ryder D, Stassi P (1999) Immobilized yeast bioreactor systems for continuous beer fermentation. Biotechnol Progr 15:105–113
38. Van Iersel MFM, van Dieren B, Rombouts FM, Abee T (1999) Flavour formation and cell physiology during the production of alcohol-free beer with immobilized *Saccharomyces cerevisiae*. Enzym Microb Tech 24:407–411
39. Wang DX, Sakoda A, Suzuki M (2001) Biological efficiency and nutritional value of *Pleurotus ostreatus* cultivated on spent beer grain. Bioresour Technol 78(3):293–300
40. Yamauchi Y, Okamoto T, Murayama H, Kajino K, Nagara A, Noguchi K (1995) Rapid maturation of beer using an immobilized yeast bioreactor. 2. Balance of total diacetyl reduction and regeneration. J Biotechnol 38(2):109–116
41. Yamauchi Y, Okamoto T, Murayama H, Kajino K, Amikura T, Hiratsu H, Nagara A, Kamiya T, Inoue T (1995) Rapid maturation of beer using an immobilized yeast bioreactor. 1. Heat conversion of α-acetolactate. J Biotechnol 38(2):101–108