Continuous primary beer fermentation with brewing yeast immobilized on spent grains

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Introduction

Various new technical, biochemical and microbiological discoveries have found application in the brewing industry during the last few decades. They are mostly used in order to achieve cost and productivity advantages in the process plant. However, the traditional beer fermentation carried out as a batch process has its own economical limitation resulting from its energy and time consuming character. The next step on the way to speed-up the wort fermentation process is considered to be its full continualisation (Pajunen, 1996).

Although the advantages of a continuous fermentation process were early recognized, there are only a few continuous brewing processes in successful operation worldwide, namely in New Zeland and Finland (Grönqvist *et al*., 1989). The reasons why continuous systems with immobilised yeasts have not yet become usual in the brewing industry include increased complexity of operations comparing to batch process, flavour problems, risk of contamination, yeast viability, carrier price and inconvenience of immobilization.

Matrixes most commonly used for yeast immobilization are gel type materials like alginate, calcium pectate and carrageenan (Dömény *et al*., 1998), porous structures namely glass beads, silicon carbide and gluten pellets (Tata *et al*., 1999; Smogrovicová *et al*., 1999) and cellulose based materials (Kronlöf *et al*., 1989; Linko *et al*., 1997). Among the latter, DEAE-cellulose and wood chips are used in full scale. Nevertheless, the need for a cheap and regenerable support material for continuous beer fermentation is still relevant.

As we have shown (Brányik *et al*., 2001) the cellulose based carrier obtained from spent grains can be considered as a promising alternative to other support materials. From an economical point of view, the spent grains are very advantageous taking into account their brewing by-product origin and simple preparation process. Further, they meet the requirements of high cell load, stability, food grade and the possibility to regenerate and sterilize. Moreover, the yeast biomass surrounding the non-porous surface of the spent grains is in direct contact with the bulk liquid reducing thus the mass transfer problems associated with other immobilized systems (Masschelein, 1997).

Besides the carrier material, the bioreactor design will also markedly influence the final success of the proposed technology. The choice of an airlift reactor for our immobilized system is in agreement with the latest trends in continuous brewing. As compared to packed-bed reactors, the systems with pneumatically forced circulation have the advantage of improved CO_2 removal, no channeling and clogging, better mass and heat transfer (Linko *et al*., 1998).

The present article deals with continuous primary beer fermentation in an airlift reactor containing brewing yeast immobilized on spent grains. Wort feed rate was optimized in terms of bioreactor performance and diacetyl formation. Different air distributors were studied in order to increase the carrier volume fraction and allow lower aeration rates.

Materials and Methods

Yeast strain and culture conditions

The brewing yeast *Saccharomyces uvarum* was supplied by a brewing company (Unicer, SA). The yeast for inoculation of the continuous airlift reactor were cultivated in 500 ml of synthetic medium under aerobic conditions on a rotary shaker (120 rpm) at 30 ºC for 30 h. The composition of the synthetic medium was as follows ($g.l^{-1}$): 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 with the same composition was used in continuous experiments during biomass attachment. The wort used in this work had an original gravity of 14 % w/w and was supplied by Unicer, SA.

Carrier preparation

Dry spent grains (100 g) were mixed in 1500 ml of 3 % (v/v) HCl solution at 60 ºC for 2.5 hours in order to hydrolyse the residual starchy endosperm and embryo of the barley kernel present in the spent grains. The mixture was cooled, washed with water and dried. The remaining solids (ca. 30 g), mainly the husks of the barley grain, were partially delignified by shaking (120 rpm) in 500 ml of 2 % (w/v) NaOH solution at 30 ºC for 24 hours. After being several times washed with water until neutral pH and dried, the carrier (ca. 10 g) was ready to be used. The preparation procedure gives 10 % (w/w) yield from dry spent grains. The drying steps applied in the preparation procedure were necessary only in order to quantify the yields.

Reactor systems

Airlift reactor (ALR). The ALR used in this work is of the concentric draught tube type with an enlarged top section for degassing and total working volume of 6 liters. The dimensions of the reactor are: total height - 90 cm; down comer's length - 60 cm, inside diameter - 7 cm; draught tube's length - 56 cm, diameter - 3.2 cm, thickness - 4 mm; cylindrical part's length - 14.5 cm, diameter - 19.2 cm. The angle between the conical sector and the main body is of 51°. The air injection is made either by means of a nozzle injector with a diameter of 1 mm immediately below the annulus of the riser or by perforated plate with 7 holes each of 1 mm in diameter and 2.5 cm below the annulus of the riser. The outflow of the reactor was placed behind a sedimentation barrier minimizing carrier losses. The temperature inside the reactor was maintained by means of a cooling coil connected to a refrigeration bath. Air flow rate was kept constant using a mass flow controller (Hastings).

ALR system startup. The ALR reactor made of Plexiglas was before utilization sterilized by sodium hypochlorite solution (2 % active chlorine) during at least 4 days. After draining the reactor the sterile air supply was started, the driving force of the liquid circulation, and the reactor was filled with sterilized slurry of the spent grains (120 g in dry state) in distilled water (3 liter). Prior to inoculation, the reactor containing fresh carrier was washed with 50 l of sterilized water. Subsequently, the reactor was charged with concentrated medium to obtain the desired concentration of the synthetic medium and then inoculated by 1 liter of yeast cell suspension grown on a rotary shaker. At the end of 24 h batch growth the feed of synthetic medium started and it took approximately 7 days at $D = 0.2\ h^{-1}$ to reach a sufficiently high immobilized biomass load (min. 250 mg dry cell g^{-1} dry carrier). Then the synthetic medium was changed to sterilized wort and the continuous system was considered to be in steady state conditions after a period of 5 residence times.

Bubble column reactor. Experiments with yeast immobilization in wort and synthetic medium were carried out in a continuous bubble column reactor with total working volume of 440 ml. The carrier (6 – 7 g dry weight) was placed in the reactor with wort or synthetic medium (10 $g\ l^{-1}$ glucose) and inoculated with 100 ml of pre-cultured brewing yeast suspension. The continuous

feed started after 16 hours of batch growth. The medium was supplied at the bottom of the reactor by means of a peristaltic pump (Watson Marlow 101 U/R). The bubble column was constantly aerated with 0.9 $l.h^{-1}$ of sterile air. The reactor outlet was connected to an overflow.

Analytical methods

Characterization of wort, green beer, measurement of free amino acids and diacetyl was performed according to the methods of EBC (European Brewery Convention).

Immobilized biomass determination. A sample containing approximately 1.0 g dry biocatalyst was taken from the reactor. The bulk liquid was removed with a syringe and the carrier was washed with 200 ml of distilled water. The carrier was filtered and washed with 400 ml of distilled water on a paper filter and then dried at 105 °C for 12 hours. An amount of approximately 0.5 g dry biocatalyst was weighed into an Erlenmeyer flask with 100 ml of 3 % wt. NaOH solution and was shaken at 120 rpm for 24 h. During this time the attached biomass was completely removed from the carrier, as verified under the microscope. The biomass free carrier was filtered and after being carefully washed on the filter with 400 ml of distilled water it was dried at 105 °C for 5 hours. The amount of yeast biofilm was determined from the weight difference before and after the treatment with caustic. Corrections of the biomass weight for the losses of carrier itself were carried out by blank experiments with clean carrier.

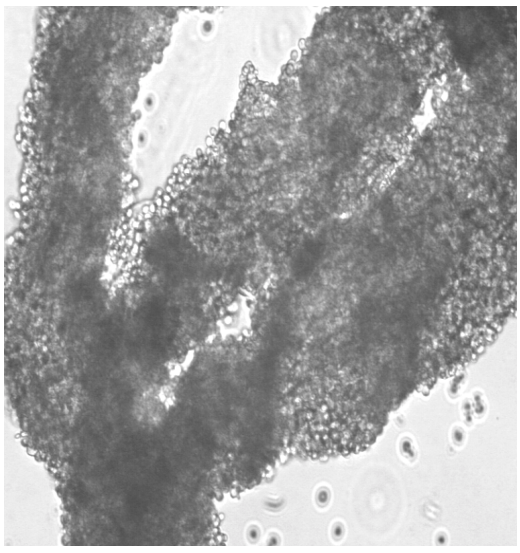
Holocellulose determination. The carrier made of spent grains (cca. 5 g) was soaked in water (160 ml). Then acetic acid (10 drops) and sodium chlorite (1,5 g) was added and the mixture was kept at 80 – 90 °C during 1 hour. The heating with preceding addition of acetic acid and $NaClO_2$ was repeated three times. The isolated white holocellulose was filtered, washed with water and acetone and weighted after drying at 105 °C.

Results and discussion

Adhesion of brewing yeast on the surface of spent grains

We suppose that at the beginning of the continuous experiment, namely during the batch growth preceding the start of the medium flow and during the first days of the continuous reactor operation, spontaneous immobilization takes place. The carrier made of spent-grains, although containing approximately 90 wt. % of holocellulose, can neither be considered chemically uniform

A



B

Fig.1: A - photograph of the sheet-like carrier obtained from spent grains; B - photograph of the carrier with attached yeast cells.

nor having homogeneous surface properties. The yeast population in the continuous system cannot be considered homogeneous alike. Therefore, the contact between the surfaces of the spent grains with yeast surfaces might result in a spontaneous and energetically advantageous interaction leading to a stabile cell adhesion. Microscopic observations from the beginning of the immobilization revealed a non-homogeneous biomass distribution all over the carrier surface with local biomass accumulations confirming thus the existence of preferred attachment sites. Starting from these initial colonies the yeast gradually covered the whole surface of carrier particles with a biofilm of variable thickness reflecting the differences in the carrier's surface properties (Fig. 1.). However, it must be also said that, although the carrier has dominantly a plain sheet or thread-like shape, a spatial retention of yeasts by tangled carrier particles can enhance local biomass accumulation as well.

Immobilization of brewing yeast in wort

The immobilization of yeast in the airlift reactor was carried out by feeding it with synthetic medium before starting the beer fermentation experiment. This start-up period of the reactor was characterised by high medium consumption and therefore the choice of the synthetic medium allowed us to avoid difficulties with supply or storage of wort.

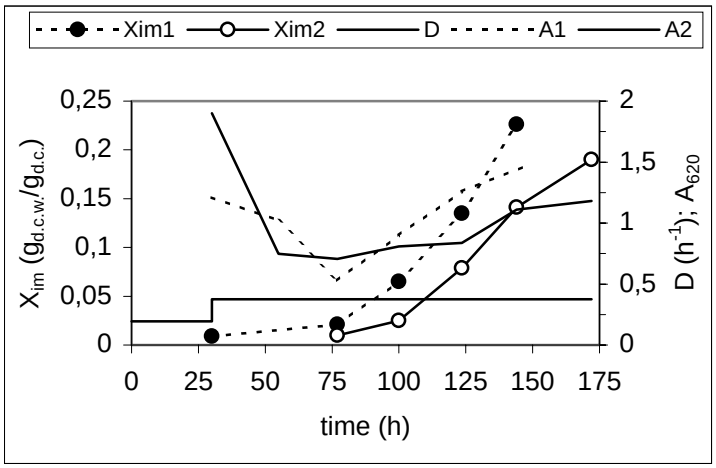


Fig. 2. Development of yeast biofilm X (g dry cell weight g^{-1} dry carrier) on the surface of spent grain and absorbance of free cells (A_{620}) in: 1 – synthetic medium, 2 – wort; at a maximum dilution rate D = 0.375 h^{-1}.

However, experiments in a bubble-column reactor proved that the attachment of brewing yeast to spent grains occurred also in wort as a real beer fermentation medium. In Figure 2. we compared the course of the yeast adhesion in synthetic medium and wort at the same dilution rate profile. Although the onset of the yeast attachment in wort was delayed by 1 day and the immobilized biomass load (X_{im}) reached in average by 25 % lower values comparing to the synthetic medium, the immobilization under beer fermentation conditions can also be considered satisfactory.

Immobilized biomass load and free cell growth

The initial startup period of the continuous experiments in airlift reactor (ALR) carried out in synthetic medium as a feed gave rise to a spontaneous attachment of brewing yeast to the surface of spent grain particles. After a sufficient immobilized biomass load was obtained, approximately 0,3 $g_{\text{d. cell}}\ g^{-1}_{\text{d. c.}}$, the reactor feed was changed to wort (9^{th} day), which further increased the amount of immobilized biomass (0, 48 $g_{\text{d. cell}}\ g^{-1}_{\text{d. c.}}$). Simultaneously, the change to a fermentation medium with higher substrate concentration (wort) resulted in an increased free biomass concentration (Fig. 3.). The cell viability remained constant throughout the duration of the experiment showing no more than 3 % of dead cells in the outflow (data not shown).

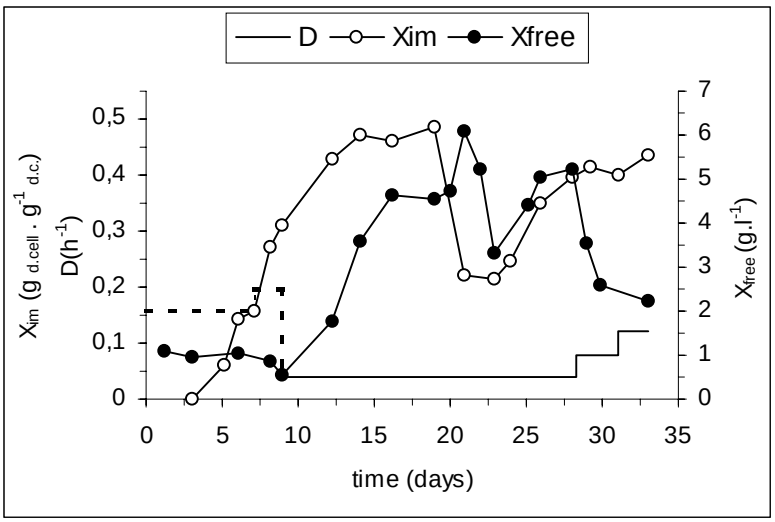


Fig. 3. Immobilized and free cell concentration during main fermentation: O - immobilized biomass load (g dry cell.g^{-1}dry carrier); ● - free cell concentration (g.l^{-1}); dilution rate of synthetic medium (dashed line) and wort (solid line); temperature – 12,5 °C, aeration rate - 0,8 l min^{-1}

Cell loading on the immobilization support is an important variable of the immobilized cell reactor system. From the values of the immobilized and free biomass (Fig. 3.) and the amount of carrier in the reactor, approximately 100 g of dry carrier, it appears that the ratio between immobilized cells and free cells in the reactor was about 1,7 – 2,0 at the dilution rate D = 0,04 h^{-1}. This is in good agreement with the ratio reached in a two stage fluidized bed system although at lower dilution rate of ca. 0,026 h^{-1} (Tata *et al.,* 1999). At higher wort feed rates the immobilized/free cell ratio was shifted to values between 2,8 and 3,0 due to a sharp drop in the concentration of free cells caused by wash out, while the immobilized cell load remained either constant or had a slightly rising tendency (Fig. 3.).

Attempts to increase the proportion of immobilized cells by using more support material in the reactor resulted in intensive carrier wash out until an equilibrium carrier volume fraction around 1,5 – 1,8 % w/v (90 – 105 g dry carrier per reactor volume) was formed. This amount of swollen spent grains plus the additional fully developed yeast biofilm on its surface represents approximately 25 % vol. of solid fraction in three-phase ALR bioreactor. Therefore, further increases of carrier (solid) volume fraction (>2 % w/v) caused an appearance of dead zones in the reactor. Especially important was the one below the sedimentation barrier of the outflow, with insufficient mixing. The lack of the liquid mixing below the outflow caused a formation of floating carrier particle clumps, which due to poor CO_2 removal comprised gas bubbles, leading thus to biocatalyst losses through the outflow. Significant carrier losses (ca. 0,5 – 1,0 g dry carrier h^{-1}) continued until the solid volume fraction decreased to ca. 1,5 – 1,8 % w/v and then the particle agglomeration and flotation diminished. Although accidental carrier wash out was observed during the whole continuous main fermentation it is of little importance since the carrier can be repeatedly replaced during long-term reactor operation. Better liquid mixing, biocatalyst homogenization and CO_2 removal allowing us to use higher carrier volume fraction can be achieved by increasing the driving force of the mixing. Therefore, perforated plate was preferred to nozzle injector as an air distributor, since it showed shorter mixing times in the whole range of studied aeration rates. However, as a consequence of the increased air supply an excessive biomass growth, undesirable flavor changes and foaming would prevail over the advantage of higher biocatalyst content.

On the 19th day of the continuous fermentation a 12 hours long break in the air supply caused that the carrier settled on the bottom of the reactor and in order to re-establish the circulation it was necessary to vigorously agitate the ALR. This mechanical stress together with the changed physiological conditions caused a considerable release of the immobilized biomass increasing temporarily the free biomass concentration in the outflow (Fig. 3.). It took about one week until the biomass balance in the ALR returned to the state comparable with the one before the aeration failure. Nevertheless, it proved the self-regulatory abilities of the continuous immobilized yeast bioreactor system to overcome the adverse effect of process failures.

Influence of the feed rate on main fermentation performance

The fermentation performance of our immobilized yeast bioreactor can be described using the values of apparent and real degree of attenuation. These parameters represent the percentage of the consumed fermentable sugars non-corrected and corrected with respect to the interference of the produced ethanol. The optimal dilution rates (D) or residence times (RT) of the reactor have been found experimentally by variation of the wort feeding rate. The desired degree of apparent attenuation for one stage primary beer fermentation, i.e. 70 – 80 %, was obtained in the range of D and RT of approximately D = 0,04 – 0,055 h^{-1} and RT = 18 – 25 hours, respectively (Fig. 4.). At these feeding rates the apparent extract of the green beer is about 3 - 4 % w/w (Fig. 4.), containing still ca. 10 % of fermentable sugars that can be used as substrate by the yeast during secondary fermentation. The efficiency (measured by degree of attenuation) and the productivity (in terms of green beer production) of the main beer fermentation in ALR with yeast immobilized on spent grains was in the same range as published elsewhere (Tata *et al.,* 1999; Virkajärvi and Kronlöf, 1998; Smogrovičová *et al*., 1997)

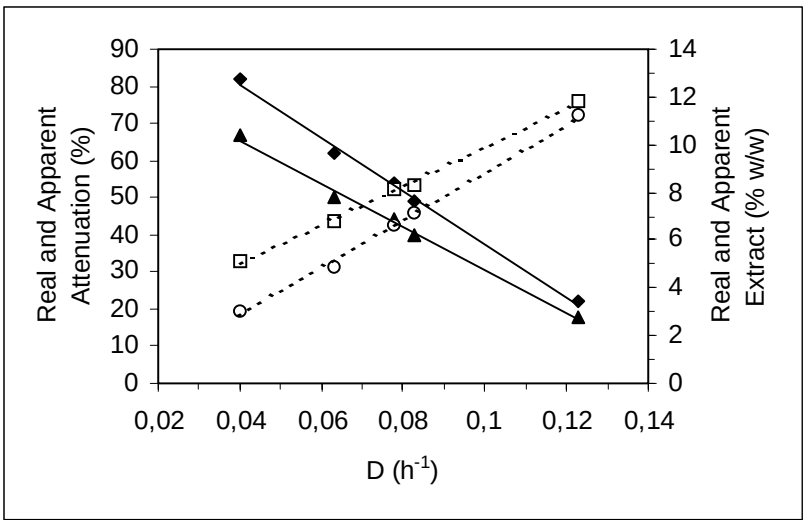


Fig. 4. The influence of the wort feed rate on selected parameter of the main fermentation: ▲ - real attenuation (%); ◆ - apparent attenuation (%); □ - real extract (% w/w); O - apparent extract (% w/w)

Besides the fermentable sugars being consumed, there are several compounds produced during the main fermentation, which strongly influence the flavor profile of the future beer. At the degree of the apparent attenuation around 70 – 80 % the concentration of ethanol produced in ALR was 3,8 - 4,5 % w/w (Fig. 5.). Taking into consideration the high original gravity of the wort used in this work (14 % w/w) it is not surprising that the ethanol concentration of the green beer produced by ALR is somewhat higher than in the studies published so far (Dömény *et al.*, 1998; Smogrovičová *et al*., 1997; Kronlöf *et al.*, 1988).

Biomass growth during fermentation is accompanied by accumulation of diacetyl that gives rise to an undesirable "buttery" flavor in the final product. Diacetyl is formed by spontaneous decarboxylation of α-acetolactate, an intermediate of the valine – leucine biosynthetic pathway, and is subsequently re-assimilated by the yeast. However, at short hydraulic residence times in continuous fermentation, the reduction of diacetyl and its precursor is not complete. As a result of an intensive biomass growth in ALR high levels of diacetyl were measured in the whole range of D (Fig. 5). At the lowest studied D the diacetyl concentration in green beer was 0,32 mg/l being approximately three times more than the threshold in mature beer. In consequence of this a biomass growth control by aeration rate and process temperature optimization should be carried out and/or an accelerated diacetyl (precursor) conversion and maturation system would have to be applied (Hyttinen *et al*., 1995)

Another indicator of the excessive biomass growth in ALR was the high free amino nitrogen (FAN) consumption. The initial concentration of FAN in wort, i.e. 250 mg/l, decreased at D = 0,04 h^{-1} to ca. 45 mg/l (Fig. 5.) that is a value lower than those found in the literature. Keeping in mind,

that the origin of the diacetyl precursor is in amino acid metabolism and that various intermediates and byproducts of amino acid metabolism are organoleptically important, the high FAN (amino acid) uptake by yeast is gaining importance.

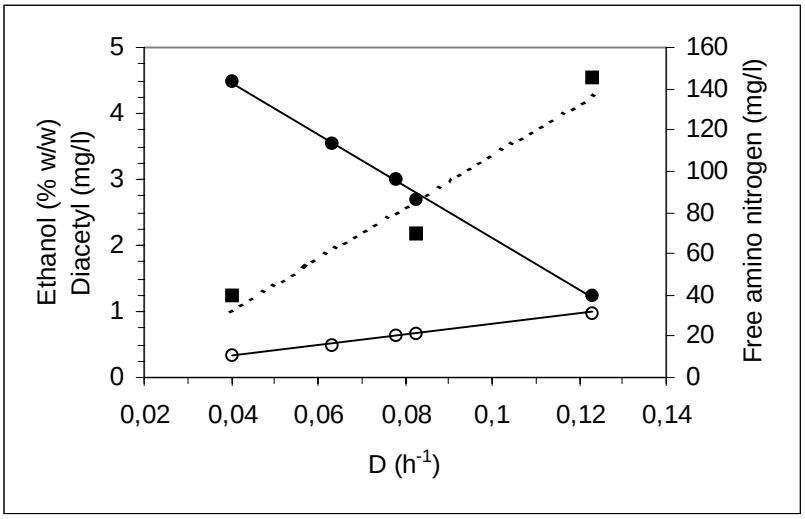


Fig. 5. The influence of the wort feed rate on selected parameter of the main fermentation: ● - ethanol (% w/w); O - diacetyl (mg/l); ■ - free amino nitrogen (mg/l)

Conclusions

These results demonstrate the technological feasibility of the three-phase airlift bioreactor with brewing yeast immobilized on spent grains for continuous beer production. We showed that, besides the fermentation process, also the start-up period could be carried out in wort as a substrate. The productivity of the system in terms of dilution rate, degree of attenuation (fermentation) and ethanol production were fully comparable with the literature. The immobilized yeast fermentation in ALR turned out to be very robust in recovery after process upsets as well. Although the diacetyl formation was higher than in the traditional technology, its level can be reduced by cell growth control, aeration and temperature optimization. We assume that especially the air input, the driving force of the circulation in ALR, could be to large extent replaced by CO_2 (Virkajärvi and Kronlöf, 1998) in order to reduce excessive cell growth. The one stage process applied in this work can be also replaced by a two or multi stage system where the cell growth and substrate metabolism takes place in distinct reactors imitating thus the conditions of the traditional batch fermentation (Andries *et al*., 1995). At last, we believe that the ALR system with brewing yeast immobilized on the surface of cellulose based carrier made of spent grains (brewing by product) is a promising alternative to existing fermentation systems.

Ackowledgement

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Literature

Andries M, van Beveren P, Goffin O, Masschelein C (1995) Design of a multi-purpose immobilized yeast bioreactor system for application in the brewing process. *Immobilized yeast in the brewing industry*; Monograph XXIV, *Eur. Brew. Conv. Symposium*. Espoo, pp. 134 - 144

Brányik T, Vicente AA, Machado Cruz JM, Teixeira JA (2001) Spent grains - a new support for brewing yeast immobilisation. *Biotechnol. Lett.* **23**: 1073-1078

Dömény Z, Šmogrovicová D, Gemeiner P, Šturdík E, Pátkova J, Malovíková A (1998) Continuous secondary fermentation using immobilised yeast. *Biotechnol. Lett.* **20**: 1041–1045

Grönqvist A, Pajunen E, Ranta B (1989) Secondary fermentation with immobilised yeast - industrial scale. In: *Proc.Eur. Brew. Conv.* Zurich, pp. 339-346

Hyttinen I, Kronlöf J, Hartwall P (1995) Use of porous glass at Hartwall brewery in the maturation of beer with immobilized yeast. *Immobilized yeast in the brewing industry*; Monograph XXIV, *Eur. Brew. Conv. Symposium*. Espoo, pp. 55 - 61

Kronlöf J, Härkönen T, Hartwall P, Home S, Linko M (1989) Main fermentation with immobilised yeast. In: *Proc.Eur. Brew. Conv.* Zurich, pp. 355–362

Linko M, Virkajärvi I, Pohjala N, Lindborg K, Kronlöf J (1997) Main fermentation with immobilized yeast - a breakthrough? In: *Proc.Eur. Brew. Conv.* Maastricht, pp. 385–394

Linko M, Haikara A, Ritala A, Penttilä M (1998) Recent advances in the malting and brewing industry. *J. Biotechnol*. **65**: 85–98

Masschelein ChA (1997) A realistic view on the role of research in the brewing industry today. *J. Inst. Brew*. **103**: 103–113

Pajunen E (1996) The behaviour of immobilised yeast cells. *Cerevisiae*. **4**: 33–37

Smogrovicová D, Dömény Z, Slugen D, Pátkova J, Bafrncová P (1999) Gluten pellets to immobilise yeast for brewery fermentations. *Monatsschrift für Brauwissenschaft* **7/8**: 119–122

Tata M, Bower P, Bromberg S, Duncombe D, Fehring J, Lau V, Ryder D, Stassi P (1999) Immobilised yeast bioreactor system for continuous beer fermentation. *Biotechnol. Prog.* **15**: 105–113

Virkajärvi I, Kronlöf J (1998) Long-term stability of immobilized yeast columns in primary fermantation. *J. Am. Soc. Brew. Chem.* **56**: 70-75