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IMMOBILIZED CELL TECHNOLOGY IN BEER BREWING — CURRENT EXPERIENCE AND RESULTS*

ABSTRACT: Immobilized cell technology (ICT) has been attracting continual attention in the brewing industry over the past 30 years. Some of the reasons are: faster fermentation rates and increased volumetric productivity, compared to those of traditional beer production based on freely suspended cells, as well as the possibility of continuous operation. Nowadays, ICT technology is well established in secondary fermentation and alcohol-free and low-alcohol beer production. In main fermentation, the situation is more complex and this process is still under scrutiny on both the lab and pilot levels.

The paper outlines the most important ICT processes developed for beer brewing and provides an overview of carrier materials, bioreactor design and examples of their industrial applications, as well as some recent results obtained by our research group. We investigated the possibile applications of polyvinyl alcohol in the form of LentiKats®, as a potential porous matrices carrier for beer fermentation. Given are the results of growth studies of immobilized brewer's yeast *Saccharomyces uvarum* and the kinetic parameters obtained by using alginate microbeads with immobilized yeast cells and suspension of yeast cells as controls. The results indicate that the immobilization procedure in LentiKat® carriers has a negligible effect on cell viability and growth. The apparent specific growth rate of cells released in medium was comparable to that of freely suspended cells, implying preserved cell vitality. A series of batch fermentations performed in shaken flasks and an air-lift bioreactor indicated that the immobilized cells retained high fermentation activity. The full attenuation in green beer was reached after 48 hours in shaken flasks and less than 24 hours of fermentation in gas-lift bioreactors.

KEY WORDS: beer, immobilized yeast, gas-lift bioreactor, alginate beads, PVA LentiKat® carriers

INTRODUCTION

Brewing is one of the oldest biotechnologies with the history dating back more than 8000 years. For centuries, people were brewing beer empirically, using the old recipes. Then, in the 18th century, the biologists began to study

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the process of brewing and to discover some of its basic principles. These early discoveries led to a better understanding of the process and, ultimately, to the development of the brewing industry as we know it today. Since the Second World War, the brewing industry, like all other branches of the processing industry, began to utilize larger production units and to reduce production costs.

Fermentation is the essential part of the brewing process. The main fermentation is responsible for the formation of most flavor compounds, while the secondary fermentation provides beer maturation and final beer sensory properties. These are, at the same time, the most time consuming steps in the overall beer production. From the very beginning of the modern brewing age, science and technology have had an important influence on the development of the novel technical solutions and the improvement of the existing ones. They led to the design of new accelerated fermentation methods (which incorporate improved batch bioreactors, ranging from open, relatively shallow tanks to large cylindro-conical fomenters) and to the development of continuous beer fermentation processes.

Since the beginning of the 20th century, many different systems that use suspended yeast cells have been developed. First fully continuous process for beer fermentation (van Rijn), with six vessels in series, was patented in 1906. During the 1950s and 1960s, several of them have been in commercial use. They had clear advantages over the traditional batch techniques: lower investment costs, lower extract losses, lower fuel and power consumption and, finally, billing practice, etc. (B i s h o p, 1970; T h o r n e, 1968). However, they were not commercially successful due to many practical disadvantages. Depending on the system in use, they caused numerous problems. The list of disadvantages was very long: inflexibility in the output rate or in the ability to change beer type, flavor-matching, need for a high standard of hygiene, possibility of yeast mutation, need for an extra procedure, need for an extremely flocculent yeast, wort storage requirements, cost of technical support, etc. (T h o r n e, 1968). Therefore, by the end of the 1970s most of the operating systems were closed down. The famous exception is the Coutts' process in Dominion Breweries, New Zealand (H o u g h, 1982). It was obvious that the right solution had to be found. Immobilized processes were expected to be a viable one.

APPLICATION OF IMMOBILIZED CELL TECHNOLOGY
IN BREWING INDUSTRY

Traditional beer fermentation technology uses freely suspended yeast cells to ferment wort in a non-stirred batch reactor. The primary fermentation for lager beer takes approximately 5 to 7 days with a subsequent secondary fermentation (maturation) of several weeks. Modern batch fermentation technology can reduce the production time (main and secondary fermenation) of lager beer to 10—12 days, but this is the best it can do. Immobilized cell technology (ICT) is able to produce lager beer in less than 2 days.

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ICT processes have been designed for different stages in the beer production/fermentation process. The most important of these are: bioflavouring during the maturation, main fermentation and fermentations for the production of alcohol-free or low-alcohol beers. The main objective of flavour maturation is the removal of the vicinal diketones diacetyl and 2,3-pentanedione, and their precursors a-acetolactate and a-acetohydroxybutyrate. Diacetyl is reduced by yeast reductases to 2,3-butanediol via acetoin and 2,3-pentanedione to 2,3-pentanediol via acethylethylcarbinol. The conversion of the α-acetohydroxy acids to the vicinal diketones in traditional maturation process is the rate limiting step. It is characterized by a near-zero temperature, low pH and low yeast concentration, resulting in a maturation period of 3 to 4 weeks. Using immobilized yeast cells this period can be reduced to 2 hours. The reaction step is accelerated by heating the beer after yeast separation to 80—90°C during a period of a few minutes (P a j u n e n, 1995; B a c k et al., 1998).

The traditional technology to produce alcohol-free or low-alcohol beer is based on the suppression of alcohol formation by restricted batch fermentation or on the removal of ethanol, using membrane, distillation or vacuum evaporation processes (M u l l e r, 1990; N a r z i s s et al., 1992). In both cases, the problem is either the undesirable wort aroma from wort aldehydes, which are reduced only to a limited degree, or the insufficient process selectivity. A short-contact with the immobilized yeast cells at a low temperature solves these problems (M e n s o u r et al., 1997; N a v r á t i l et al., 2000).

The design and optimization of an ICT process for the combined main and secondary fermentation remains a challenging task. In spite of much experimental work, ICT processes have not yet been adopted in the brewing industry. When compared to the secondary fermentation, the main fermentation is significantly more complex and has various side reactions that are important for final beer quality. It was reported that insufficient free amino nitrogen consumption by immobilized yeast cells, coupled with mass transfer restrictions and reduced cell growth in immobilized conditions, causes an unbalanced flavor profile of the final beer. Immobilized systems, based on packed bed fermenters with solid carriers for yeast cells and suitable for secondary beer fermentation, were shown to be inappropriate. The reasons include flavor problems, yeast viability and carrier price.

PROCESS DEVELOPED FOR BEER FERMENTATION

The brewing industry has been showing interest in ICT since its appearance and particulary after the introduction of alginate as a carrier (W h i t e and P o r t o, 1978). One of the first processes for rapid lager fermentation was developed by the research team at „Kirin Brewery Company", Japan. It was a multistage ICT system for fermentation and maturation. The process consisted of three bioreactors. The first was an aerated continuously stirred tank for yeast growth. It was followed by two packed bed reactors, in series, for main fermentation and heat treatment for a-acetolactate conversion into diacetyl. Finally, there was a packed bed reactor with immobilized yeast for maturation.

Using this process, beer could be produced within three to five days. The first immobilization method that was used was entrapment in alginate beads, but because of the problems it caused (decreased fermenting capacity, insufficient mechanical strength, swelling of the carrier leading to plugging of the bioreactor, etc.) it was replaced by ceramic beads developed by Kirin („Bioceramic®") (Y a m a u c h i, 1994). The Company set up a small commercial unit on the Saipan island, producing 1850 hl per year. However, their brewing proved to be short lived. The lager beer they produced was sensorly acceptable, but somewhat different from the conventional batch beer. In addition, they could not reach one fifth of the designed output capacity without experiencing deterioration in yeast fermentation activity. Finally, the energy costs and the beer losses were high with centrifuged yeast (I n o u e, 1995).

A research group at „Labatt Breweries" (Interbrew, Canada) in collaboration with the Dept. of Chemical and Biochemical Engineering at the University of Western Ontario went in another direction. They applied k-carrageenan beads as a carrier in a draft tube bed reactor. The advantage of k-carrageenan as the carrier material is its density, which is close to that of water and thus minimizes the energy required for fluidization. Small bead size (0.2 to 1.4 mm), fluidized bed design (feed gas mixture of 2—5% of air and CO_2) and a better mass transfer were expected to solve the problems with insufficient amino acid consumption and unbalanced flavor profile. Pilot scale research showed that in continuous fermentation, full attenuation was reached in 20—24 hours and the flavor profile of the beer was acceptable and similar to the batch fermented beer (M e n s o u r et al., 1995; P i l k i n g t o n et al., 1999).

„Meura Delta", Belgium, proposed a completely different concept. Their goal was to solve the problem of unsuitability of alginate beads in a packed bed reactor and carrageenan beads in a fluidised bed reactor and so they developed a tubular matrix of sintered silicon carbide installed into a loop bioreactor. The system has been used for maturation, alcohol-free beer production and for main fermentation (V a n D e W i n k e l, 1995; A n d r i e s et al., 2000). For the main fermentation of lager beer, two similar bioreactors were used in series. The first bioreactor was operated at an apparent attenuation level of 40%, and the complete attenuation was reached in the second bioreactor. The residence time was 22 h per bioreactor, while productivity for one matrix depended of the wort's original gravity: at 12—16° Plato, it was 6.6—9.1 hl per year, respectively. This means that for achieving an annual output of 1 million hl, more than 100000 matrices are needed (V i r k a j ä r v i, 2001).

VTT Research Institute (Finland) offered a solution for beer maturation that uses a DEAE-cellulose carrier in a fixed-bed reactor. These results led to industrial applications at the Sinebrychoff's Helsinki brewery in 1990 and later on at the Sinebrychoff's Kerava brewery, where the production levels of 1 million hl per year were achieved (P a j u n e n, 1995). Using a traditional main fermentation and heat treatment of green beer, the maturation period for diacetyl conversion has been reduced from 3—4 weeks to 2 hours. Later on, this carrier was replaced by cheaper aspen wood chips for yeast cell immobilization (V i r k a j ä r v i, 2002). Few years after that, Synebrychoff Brewery (Finland), in collaboration with Guinness, GEA Liquid Processing Scandinavia

and Cultor Corporation of Finland, developed a new ICT process for continuous main fermentation that uses a fixed-bed reactor and DEAE-cellulose at the beginning, and wood chips later on, as carrier materials. Good quality beer and constant flavor profile were achieved at a production time of 20 to 30 hours (A n d e r s e n, 1999).

„Alfa Laval and Schott Engineering" developed a maturation system based on porous glass beads. This system has been implemented in several breweries in Finland, Belgium and Germany. The produced beers had overall good analytical and sensorial properties (D i l l e n h o f e r and R o n n, 1996).

Yeast immobilized on DEAE-cellulose packed in a column reactor has been successfully applied for controlled ethanol production of low-alcohol and alcohol-free beers (V a n D i e r e n, 1995). This technology has been implemented by Bavaria Brewery (The Netherlands and several other companies, including Faxe (Denmark), Öttakringer (Austria) and a Spanish brewery (M e n - s o u r et al., 1997).

Our group started the experiments on application of ICT in beer fermentation in early 90s. The aim was to find the optimal solution for reactor design, carrier selection and immobilization techniques. Practically at the same time when Labatt research group started with their fluidized-bed fermentor with carragenan, our group was using alginate beads in the similar type of a fermentor: a three-phase gas-lift fermentor (Figure 1) (N e d o v i ć et al., 1993). A gas-lift reactor retains the advantages of fluidized-beds, such as high loading of solids and good mass transfer properties, and is particularly suitable for applications with low-density carriers. Other important characteristics of gas-lift fermenters are their simple construction, low risk of contamination, easy adjustment and control of the operational parameters, and simple capacity enlargement (N e - d o v i ć et al., 2002). We set out to systematically investigate the conditions which might influence fermentation kinetics, yeast metabolism and, lastly, the sensory profile of final beer. We focused on porous matrices carriers: medium-viscosity Na-alginate and polyvinyl alcohol in the form of LentiKats®.

Polyvinyl alcohol in the form of LentiKats® was reported as one of the promising materials for cell immobilization (J a k e l et al., 1998; J a h n z et al., 2001). LentiKats® stands for lens-shaped gel particles, which are produced by new simple gelification technique at room tem-
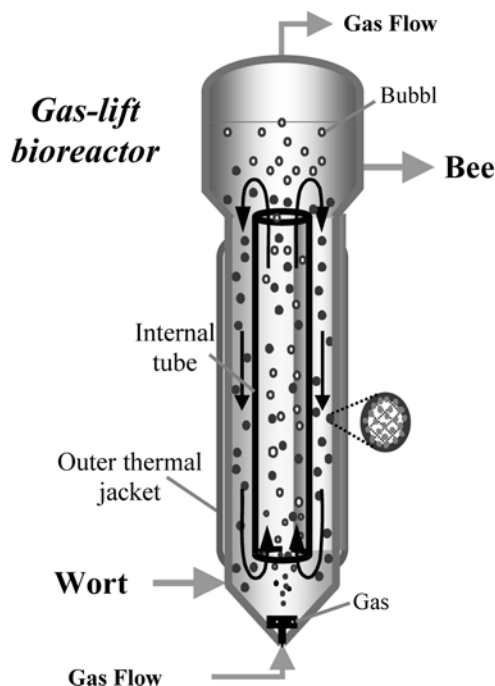

Figure 1. Gas-lift bioreactor system

perature. LentiKats® were investigated as cell carriers in several biological processes, such as bioconversion of glycerol to 1,3-propanediol (W i t t l i c h et al., 1999), cider fermentation (D u r i e u x et al., 1999, 2000, 2002), waste water treatment (S i e v e r s et al., 2002), and production of L-Tryptophane (K l a b e n et al., 2002). In the present work, we have studied the application of LentiKats® as the potential cell carriers for beer industry.

MATERIALS AND THE METHOD

2.1. *Preparation of microbeads*

Production of alginate microbeads: The technique we used to produce small enought alginate beads (< 1 mm) is the electrostatic droplet generation method. It consists of applying an electrostatic potential between the droplet formation device and the collecting solution, and inducing a charge at the surface of the polymer solution, which results in a decrease in surface tension. Using this method, a significantly greater reduction of droplet size is realized as compared to the one that is achieved using the simple dropping method (Figure 2) (N e d o v i ć et al., 2001). Process parameters were: positively charged needle set-up, applied potential: 8 kV, needle size: 27-gauge, electrode distance: 2.5 cm. Polymer/cell suspension was formed by mixing the Na-alginate solution (2% Na-alginate) with the thick brewer's yeast (*Saccharomyces uva-*
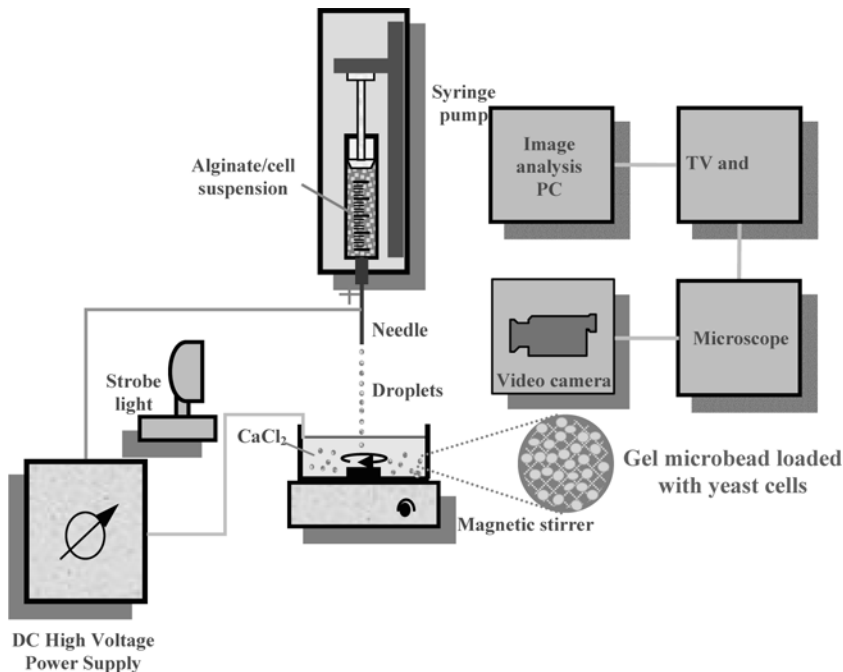
Figure 2. Production of alginate microbeads by electrostatic droplet generation

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rum). The resulting microbeads were 0.3, 0.5 and 0.6 mm in diameter and contained immobilized yeast cells at a starting concentration of 2×10^7 cells/ml.

The PVA LentiKats® were produced by a new, simple gelification technique at room temperature. With LentiKat® Printer, the PVA/yeast cell solution was forced out of the tip of a blunt edge needle (1 mm in diameter) by a syringe, in the form of droplets on Petri dishes. Gelification of the droplets occurred in approximately half an hour at a 75% decrease of the initial mass due to water evaporation. The resulting LentiKat® lenses were about 3.5 mm in diameter and 0.3 mm thick with immobilized yeast cells at a starting concentration of 1×10^7 cells/ml (Figure 3).
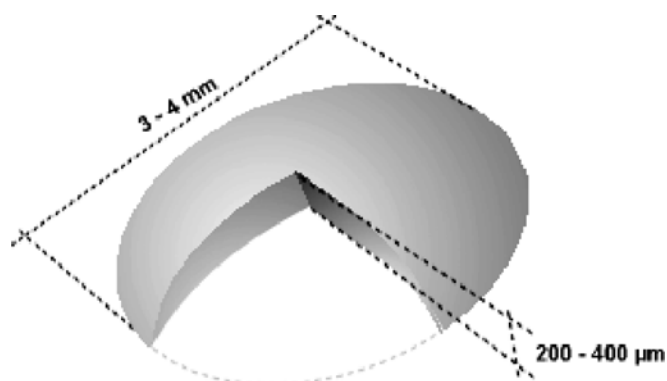

Figure 3. Schematic presentation of a LentiKat® lens

2.2. *Growth studies*

The kinetic parameters of immobilized yeast growth were investigated in a short-term study, by cultivating LentiKat® lenses for 85 hours in shaken flasks. Alginate microbeads with immobilized yeast cells, as well as the suspension of yeast cells at the same starting concentration (0.5×10^6 cells/ml) served as controls. LentiKat® lenses, alginate microbeads and the medium were sampled at timed intervals and analyzed for cell viability, concentration and colony distributions.

2.3. *Fermentation studies*

A series of batch fermentations were performed in shaken flasks and in an air-lift bioreactor. The goal was to determine the fermentation activity of brewer's yeast cells, immobilized in LentiKat® carriers, the time necessary to achieve full beer attenuation and to test the stability of LentiKat® carriers in multiple fermentations. In the first set of experiments, fermentation was managed in 500 ml flasks with 210 ml of sterile plant wort (12% extract) and 70 g of LentiKat® lenses. The experiments were performed in duplicates on an orbi-

tal shaker at 115 rpm and 17°C. The concentration of immobilized cells was about 5×10^8 cells/ml LentiKats®. LentiKat® lenses and the medium were sampled at timed intervals and analyzed. In the second set of experiments, fermentation was managed in internal-loop gas-lift bioreactor with working volumes of 1 dm³. (Figure 1). Nitrogen was introduced through a glass sparger at the bottom of the reactors, at the gas flow rate of 240 ml/min. The initial concentration of immobilized cells was about 1×10^9 cells/ml LentiKats®.

2.4. *Analytical assays*

The sizes of LentiKat® lenses with immobilized cells and the alginate microbeads were analyzed using a microscope with an accuracy of 10 mm. Cell concentrations and viabilities were determined after dissolution of lenses through heating and mixing. Yeast cell concentration was estimated with a Thoma counting chamber and the cell viability was assessed using the methylene blue-staining technique. The distribution of immobilized cells was determined by fixation of lenses and beads in 2.5% glutaraldehyde and araldite and further longitudinal- and cross-sectioning (1.5 μm). Liquid samples from both growth and fermentation mediums were collected aseptically and analyzed for specific gravity, flavor volatiles, FAN, vicinal diketones, yeast cell counts, and cell viability.

RESULTS AND DISCUSSION

Growth studies have shown that a lag phase of 22 h and an exponential growth phase of 18 h, with a specific growth rate of 0.22 h^{-1}, could be distinguished on the growth curve obtained for immobilized cells in LentiKat® carriers. Released cells were detected in the medium only after 20 h of cultivation, which approximately coincided with the start of intensive proliferation of immobilized cells (Figure 4). The increase of the cell concentration in the medium was exponential, with the apparent specific growth rate of 0.43 h^{-1}, representing the combined effects of cell proliferation in the medium and cell leakage from the carriers. The overall concentration of cells in the immobilized system (within carriers and in the medium) as a function of time was compared with the growth of yeast in free cell suspension. It was found that the immobilized cells exhibited significantly longer lag and exponential phases than freely suspended cells (22 and 18 h vs. 5 and 10 h, respectively). The apparent specific growth rate in the immobilized system was almost 2-fold lower than that obtained in the free cell suspension (0.24 vs. 0.47 h^{-1}). However, the final overall cell concentration in the immobilized system was higher than the concentration achieved in the free cell suspension due to the prolonged growth in the immobilized system (Figure 5).

The growth studies in the case of alginate microbeads showed that three general phases of microbial growth can be distinguished: a short lag phase (about 4 hours), an exponential phase (about 12 hours) and a stationary phase (until the end of the experiment) (Figure 6). The highest final cell concentra-
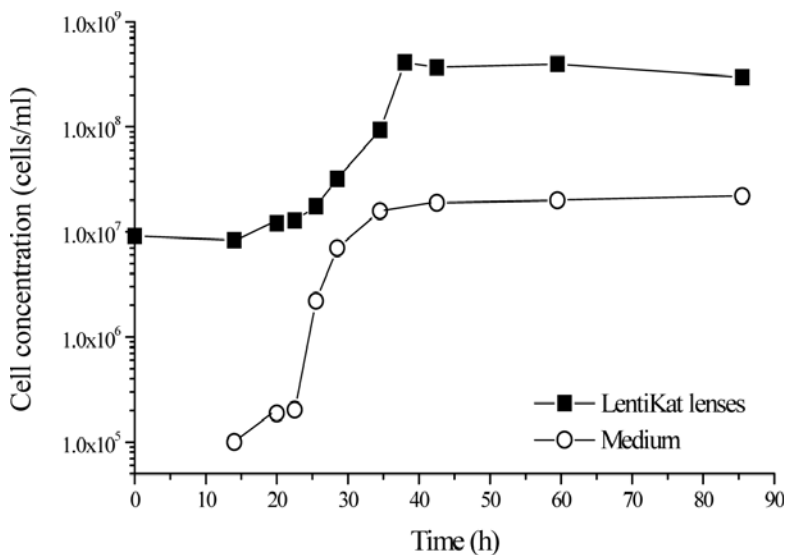
Figure 4. Growth curves in immobilized cell culture: –■– cells within LentiKat® carriers; –O– cells released into the medium.
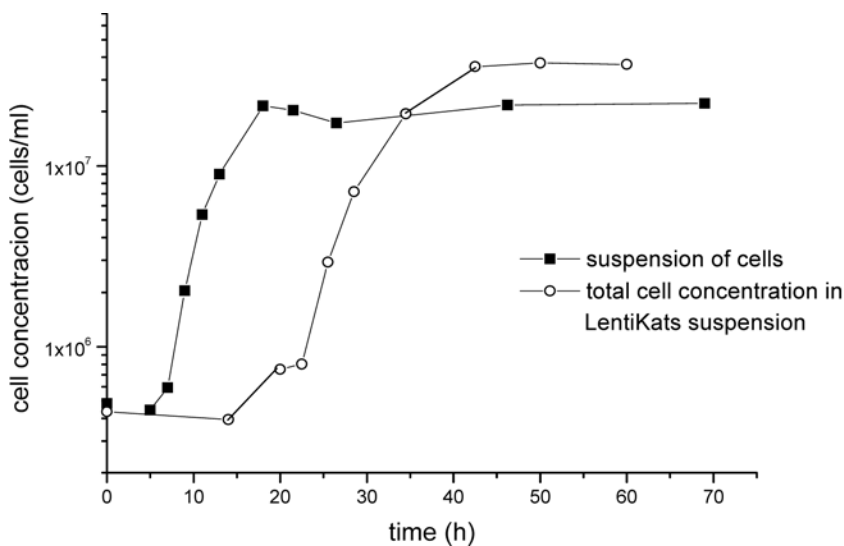

Figure 5. Comparison of cell concentrations in cell suspension and LentiKats suspension.

tion of about 2.33 x 10^9 cells/ml was found in microbeads with initial mean diameters of 0.5 and 0.6 mm.

The results of the growth studies indicated that the immobilization procedure in LentiKat® carriers had a negligible effect on cell viability and growth. The apparent specific growth rate of cells released in the medium was comparable to that of freely suspended cells, implying preserved cell vitality. In addi-
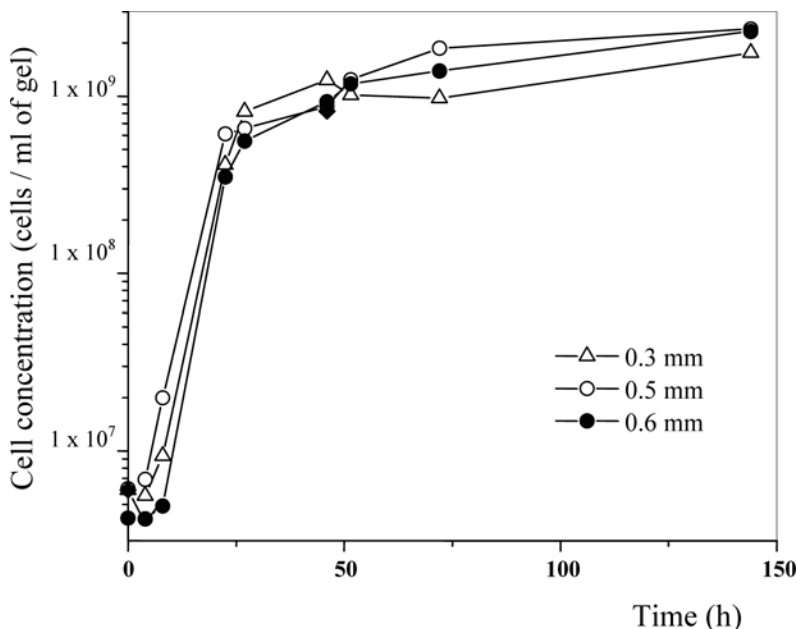
Figure 6. Yeast growth in alginate microbeads

tion, the final cell concentration achieved in LentiKat® carriers was an order of magnitude higher than the final concentration of suspended cells (5×10^8 cells/ml of carrier vs. 3×10^7 cells/ml) and a little bit lower compared to the concentrations of brewer's yeast cells in Ca-alginate microbeads.

Fermentations with yeast cells, immobilized in LentiKat® carriers in shaken flasks, showed that the apparent attenuations of around 80% were achieved after two-day fermentations. The cell concentration within LentiKat® particles stabilized at the value of around 8×10^8 cells/ml after three experimental runs, while the cell concentration in the medium was constantly increasing during fermentation runs. Constant increase of biomass production was noticed, indicating a stable functioning of the immobilized cells. High rates of biomass formation exhibited by immobilized cells could be crucial for continuous mode of application, by providing a stable source of yeast supply. The cell activity stayed constant over 4 weeks of multiple fermentations. LentiKat® particles remained intact, confirming chemical and mechanical stability. The problem noticed in these fermentation studies was significant agglomeration of particles, which resulted in the formation of clusters.

Fermentations results in the gas-lift bioreactor system were promising as well. Stable operation at gas flow rate of 240 ml/min was achieved, without agglomeration that was observed during batch fermentations in shaken flasks. The process lasted for 24 hours at relatively low solid loading (about 10%). Immobilized cells demonstrated high fermentation activity with apparent attenuation between 80 and 86%. Concentration of cells in the carrier raised from

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the initial $8 \cdot 10^8$ cells/ml to $1.4 \cdot 10^9$ cells/ml, while the final concentration in the medium was $2.1 \cdot 10^7$ cells/ml.

A batch of LentiKat® particles with immobilized brewer's yeast in shaken flasks and in gas-lift bioreactors comprised of over 60 days of operating time in a 6 month period without obvious changes in shape and size. Final beers had desired sensory and analytical profiles.

CONCLUSIONS

This study has demonstrated that LentiKat® particles could be efficiently used as carriers of brewing yeast cells in beer fermentation. The results of growth studies imply that the immobilization procedure has no adverse effects on cell viability and proliferation. Although the growth phases of immobilized cells were prolonged as compared to freely suspended cells, high final cell concentrations on the order of 1×10^9 cells/ml of LentiKats® were achieved. The immobilized cells retained such a high fermentation activity that the full attenuation in green beer was reached after 48 hours of fermentation in shaken flasks and in less than 24 hours of fermentation in the gas-lift bioreactors. Relatively low solid load was applied (10% w/v) in the gas-lift reactor, implying that even higher fermentation rates could be achieved at higher hold-ups of the solid phase. LentiKat® biocatalysts provided a stable source of yeast cells and a possibility to balance the amounts of immobilized and freely suspended cells in fermentation systems aimed at achieving high productivity and desired beer flavor.

ACKNOWLEDGEMENTS

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ТЕХНОЛОГИЈА ИМОБИЛИСАНИХ ЋЕЛИЈА У ПРОИЗВОДЊИ ПИВА — ДОСАДАШЊА ИСКУСТВА И РЕЗУЛТАТИ

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Резиме

Индустрија пива већ 30 година показује занимање за примену технологије имобилисаних ћелија. Најважнији разлози су: већа брзина и продуктивност ферментације у поређењу са традиционалном производњом пива на бази суспендованих ћелија, као и могућност континуалног рада. Данас је ова технологија већ конвенционално примењена у накнадном врењу и производњи нискоалкохолних и безалкохолних пива. Главно врење, међутим, још увек је остало нерешен проблем. Због своје комплексности, упрскос многобројним експерименталним резултатима на лабораторијском и полуиндустријском нивоу, још није реализовано на индустријском.

У раду су приказани најважнији досад развијени процеси, носачи и биореакторски системи на бази имобилисаних ћелија, примењени у различитим фазама ферментације пива, као и најновији сопствени експериментални резултати. Испитивана је могућност примене поливинил алкохола у облику LentiKats®, као потенцијалног носача за ферментацију пива. Дати су резултати испитивања кинетике раста пивског квасца *Saccharomyces uvarum* имобилисаног на LentiKats и алгинатном носачу и упоређене са вредностима добијеним за слободне суспендоване честице. Утврђено је да поступак имобилизације на LentiKat® носачу има занемарљив утицај на виталност и раст честица. Специфична брзина раста честица које се ослобађају у медијум одговарала је вредности добијеној у случају слободних суспендованих честица. Огледи у шаржним ферментацијама у тиквицама по Ерленмајеру и гас-лифт биореактору показали су да имобилисане ћелије задржавају велику ферментативну активност. Потпуна преврелост младог пива у тиквицама по Ерленмајеру достигнута је након 48 часова и за мање од 24 часа у гас-лифт биореактору.