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Immobilized cell technology, an efficient tool for producing food cultures*

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Introduction

Lactic acid bacteria (LAB) are largely used in single and mixed cultures for food fermentations, particularly for cheese, fermented milk, yogurt and cultured butter production. For production lactic starters are usually propagated by batch fermentations using freely suspended microbial cells. In this process, cell growth is limited by the accumulation of lactic acid or low pH. Continuous fermentations may overcome this limitation, but they have many drawbacks, such as their susceptibility to contamination and the appearance of undesirable genomic mutations resulting in losses of important traits (1). In addition when mixed strains are grown by continuous free-cell fermentation, dominance or wash-out of strains is usually rapidly observed; for a given dilution rate, the strain with the higher specific growth rate becomes dominant while the others are washed out from the bioreactor.

A promising alternative for continuous mixed lactic starter production or production of sensitive cultures such as probiotics is immobilized cell (IC) technology (1, 2). It has been shown that the high IC concentration maintained in the bioreactor results in very high productivity of the fermentation process (3, 4). As well, the high dilution rate and inoculation rate provided by cell release from beads lower the risk of contamination, and immobilization enhances genetic stability in recombinant or natural LAB cultures (3). In this paper, the basis of IC technology is presented and recent applications for production of mixed-strain lactic starters and probiotic cultures with altered physiology are illustrated. Further information on immobilized cell technology with LAB and probiotic bacteria can be found in recent reviews (3, 5).

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The basis of Immobilized Cell Technology

For food applications, the most widely used immobilization technique is the entrapment of cells within a food-grade porous polymeric matrix (3). Controlled-size polymer droplets immobilizing viable cells are produced using emulsification or extrusion, under mild conditions. For emulsification, a sterile polymer solution (κ-carrageenan, gellan, agarose, gelatin) previously inoculated with a small amount of cell suspension (final cell concentration in the polymer solution of ca. 10^6–10^7 CFU/mL) is dispersed under controlled mixing and temperature conditions in an hydrophobic phase (vegetable oil) (8). Thermal gelation of the polymer droplets in the macro-emulsion is used to produce 1–2 mm spherical gel biocatalysts, as illustrated in Figure 1.

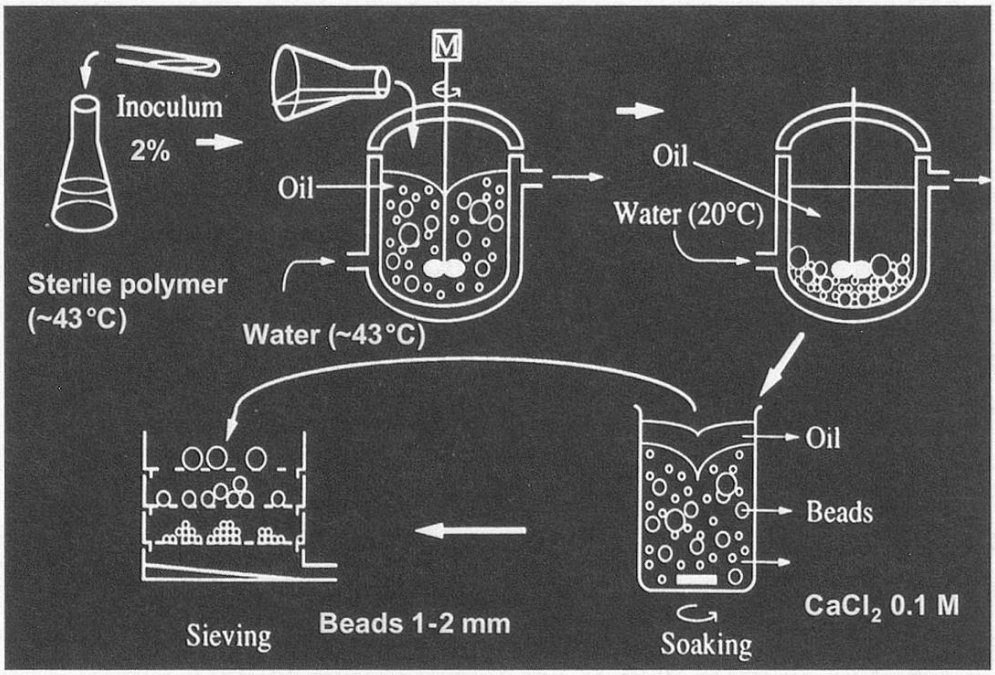

Figure 1 **Double phase dispersion process for the production of spherical gel biocatalysts with immobilized viable cells (adapted from 8)**

A careful selection of polymer composition according to the conditions of the fermentation is necessary for mechanical stability of biocatalysts during long-term continuous fermentation. For applications with LAB or for immobilization of complex microbiota, we have shown that mixed gels of deionized kappa-carrageenan/locust bean gum or gellan/xanthan gums (3 % w/w total polymer concentration) exhibited good mechanical stability during continuous fermentation for more than 3 months (3, 7).

Incubation of highly porous gel beads containing immobilized viable cells in a nutritive medium allows diffusion of nutrients from the bulk medium to the cells in beads, IC growth and counter-diffusion of metabolic products. However, growing

cells cannot freely diffuse in the gel matrix and are thus retained in beads. This results in the formation of a high cell density region (with a thickness varying from 100 to 400 µm) that extends from the bead surface to a radial position where cell growth is prevented due to lack of substrate, or accumulation of inhibitory products and low local pH (3, 10, 11) (Figure 2). In the case of LAB which are sensitive to product inhibition, product concentration and pH profiles play a major role on immobilized cell growth and productivity (9, 10).

Very high IC concentrations are measured in colonized gel beads, typically ranging from 5×10^{10} to 5×10^{11} CFU/mL or g gel (3). Cell release from gel beads in the liquid medium occurs spontaneously due to the formation of the high biomass-density peripheral layer at the bead surface with a high cell-growth activity. During continuous culture of immobilized LAB, microscopic observations showed that peripheral gel cavities containing the microcolonies are disrupted by forces resulting from cell growth and shear forces due to mechanical agitation and multiple bead contacts in the bioreactor (3) (Figure 2). Consequently, at steady state, a biofilm of fixed biomass is formed, in which cell growth and cell release in the culture medium are balanced.

The IC growth and cell release activity from the highly colonized support form the basis for biomass production in the liquid broth medium with IC technology. The very high cell density retained in the reactor (beads are kept in the reactor with a screen whereas medium is continuously fed), typically ranging from 2×10^{10} to 2×10^{11} CFU/mL, the discrete localization of immobilized cells and the high cell release from biocatalysts explain the high performances of IC technology with continuous culture compared with classical free-cell fermentations for both biomass and metabolite productions: very high volumetric productivities, high biological stability (e.g. resistance to phage and contaminants, control of mixed-strain com-

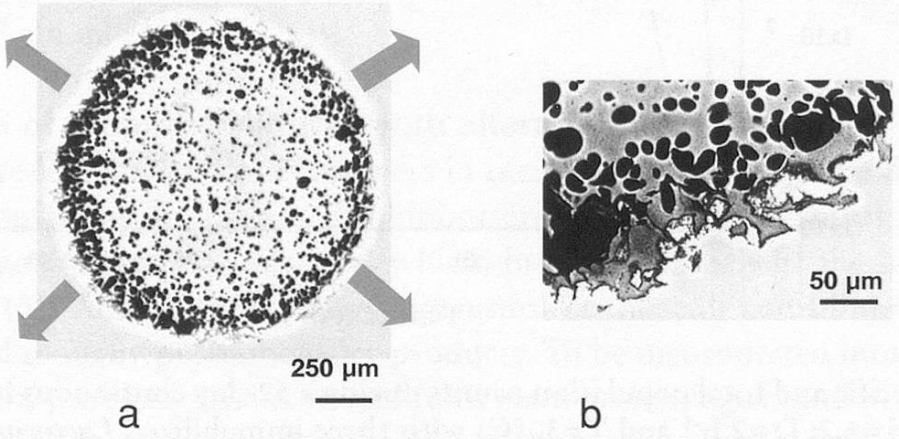

Figure 2 **Optical micrographs of immobilized cells of *Lactococcus* sp. (ca. 10^{11} CFU/g bead) in colonized gel beads.** Cells in colonies were stained with *o*-toluidine. Cell release activity (illustrated by arrows in a) results in a degradation of the bead surface (b) (adapted from 11)

plex fermentations) over long-term cultures, high flexibility, and improved process control and product yields (3).

Production of mixed-strain LAB starters

The LAB are largely used in single and mixed cultures for food fermentations, especially in the dairy industry. The use of concentrated LAB starter cultures for bulk tank or direct vat inoculation has eliminated the multiple subculture steps that were traditionally needed to build an inoculum from a mother culture. The main objectives of producing concentrated starters are to obtain a high number of living cells containing the necessary enzymes to function effectively during manufacturing of cultured milk products; and also to maintain a given strain balance in mixed starter cultures.

The production of mixed-strain mesophilic lactic starters was studied during continuous fermentations of a supplemented whey permeate medium, with three strains of *Lactococcus* sp. separately immobilized in κ-carrageenan/LBG gel beads in a stirred tank reactor (6). The process showed a high biological stability and cell productivity (mean cell productivity of 5×10^{12} CFU.L^{-1}.h^{-1}, which is 15 to 30-fold higher than that for an optimal traditional batch free-cell culture, when accounting for preparation time of the reactor) over the tested period exceeding 50 days, as illustrated in Figure 3.

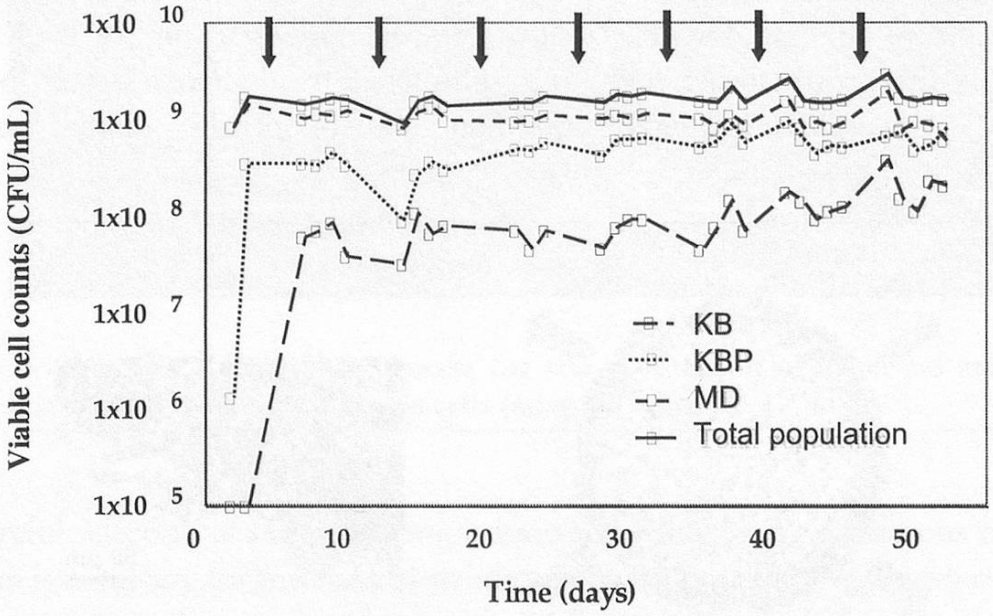

Figure 3 **Specific and total population counts during a 52-day continuous fermentation (pH=6.2; D=2 h^{-1} and T=30°C) with three immobilized *Lactococcus* sp. (KB, KBP and MD) in supplemented whey permeate medium (adapted from 6).** The arrows indicate weekend interruption of the continuous process

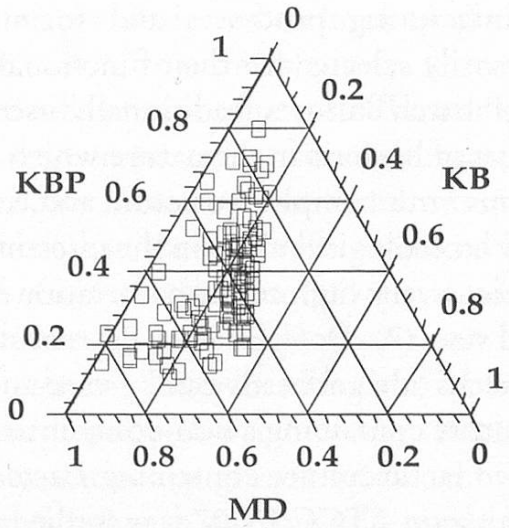

Figure 4 **Predicted starter composition (fractional composition) in the effluent of the continuous fermentation with three immobilized *Lactococcus* sp. (KB, KBP and MD) (adapted from 1)**

By varying pH, dilution rate (D) and temperature (T), a large range of strain ratios could be obtained, while starter activity remained constant or slightly increased with time (Figure 4) (1).

Doleyres et al. (12) recently studied the continuous production of *Bifidobacterium longum* ATCC 15707 immobilized in gellan gum gel beads. They showed high cell production in the fermented broth, ranging from 3.5 to 4.9×10^{9} CFU mL^{-1} for dilution rate decreasing from 2 to 0.5 h^{-1}, and maximal cell productivity of 6.9×10^{12} CFU L^{-1} h^{-1} (dilution rate of 2 h^{-1}), which represents the highest bifidobacteria maximal productivity ever reported. This productivity was approximately 9.5-fold higher than in batch free-cell cultures (productive period) at optimal pH of 5.5 (7.2×10^{8} $cfu.ml^{-1}.h^{-1}$).

Production of probiotic cultures with altered physiology

The increasing interest of consumers in their health has led to the development of foods containing probiotics. Probiotics are defined as microbial cells which transit the gastrointestinal tract and which, in doing so, benefit the health of the consumer (13). Among these micro-organisms, lactobacilli and bifidobacteria are already used in many probiotic dairy products. To be incorporated into food products, potential probiotic strains should fulfil many technological and health criteria, such as simple, large-scale production of a viable culture concentrate, survival during preparation and storage of the carrier foods, survival in the intestinal ecosystem of the host and delivery of beneficial effects after consumption (5). However, the viability and stability of probiotics has been both a marketing and technological challenge for industrial producers, since they should maintain a suitable level of

viable cells during the product's shelf life without increasing production costs. Improved or new manufacturing processes and formulation technologies are required for bacteria primarily selected for their functional health properties (5).

Conditions of free-cell batch cultures traditionally used for culture propagation are very different from that of bacteria in the nature which are usually fixed on solid supports and form biofilms with complex structure and composition. In particular, probiotic bacteria occupy an ecological niche in the intestine that is characterized by a large diversity of bacteria, a very high cell concentration and competitive environment and an immobilized state (7). *Doleyres et al.* (2) recently demonstrated that cell immobilization in polysaccharide gel beads can be used to continuously and stably produce a mixed lactic culture containing a non-competitive strain of bifidobacteria. The production of a mixed lactic culture containing *Lactococcus lactis* ssp. *lactis* bv. *diacetylactis* MD and *B. longum* ATCC 15707 was studied during a 17-day continuous IC culture at different temperatures between 32 and 37 °C. The two-stage fermentation system was composed of a first reactor (R1) containing cells of the two strains separately immobilized in κ-carrageenan/LBG gel beads and a second reactor (R2) operated with free cells released from the first reactor (Figure 5). The system allowed to continuously produce a concentrated mixed culture with a strain ratio whose composition depended on temperature and fermentation time (2). A stable mixed culture (with a 22:1 ratio of *L. diacetylactis* and *B. longum)* was produced at 35 °C in the effluent of R2, whereas the mixed culture was rapidly disbalanced in favor of *B. longum* at a higher temperature (37 °C) or *L. diacetylactis* at a lower temperature (32 °C).

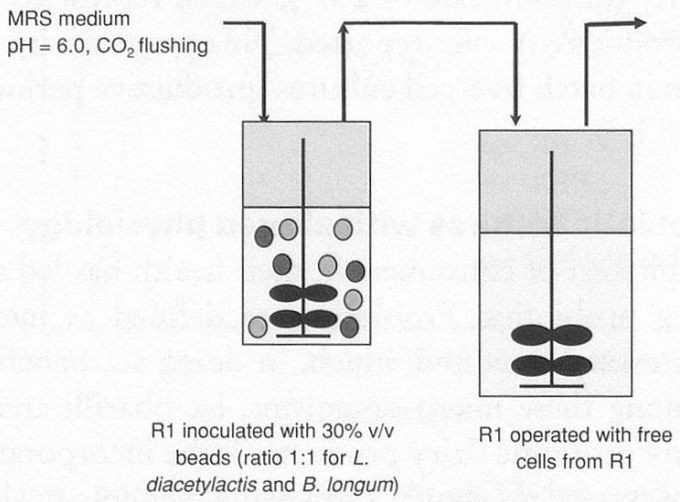

Figure 5 **Schematic representation of the two-stage continuous pH-controlled fermentation with immobilized *L. diacetylactis* and *B. longum* used by *Doleyres et al.* (2)**

Recently, *Doleyres et al.* (14) studied the effect of immobilization and long-term continuous-flow culture on probiotic and technological characteristics of cultures produced in the effluent medium from the two-stage fermentation system shown in Figure 5. Tolerance of free cells produced in the effluent medium to various stresses including freeze-drying, oxygen peroxide, simulated gastro-intestinal conditions, nisin, and antibiotics markedly increased with culture time and were generally significantly higher after 6 days than those of cells produced by conventional free-cell batch fermentations. This effect of cell immobilization and time on stress resistance of *B. longum* is illustrated for cell survival in gastric and intestinal juice (Figure 6) and for tolerance of *B. longum* to selected antibiotics (Table 1) and *L. diacetylactis* to kanamycin (Figure 7). In addition, cells produced by continuous IC cultures, which are in exponential or early-stationary growth phase, exhibit both a high viability and metabolic activity compared with starving cells produced by conventional free-cell batch cultures.

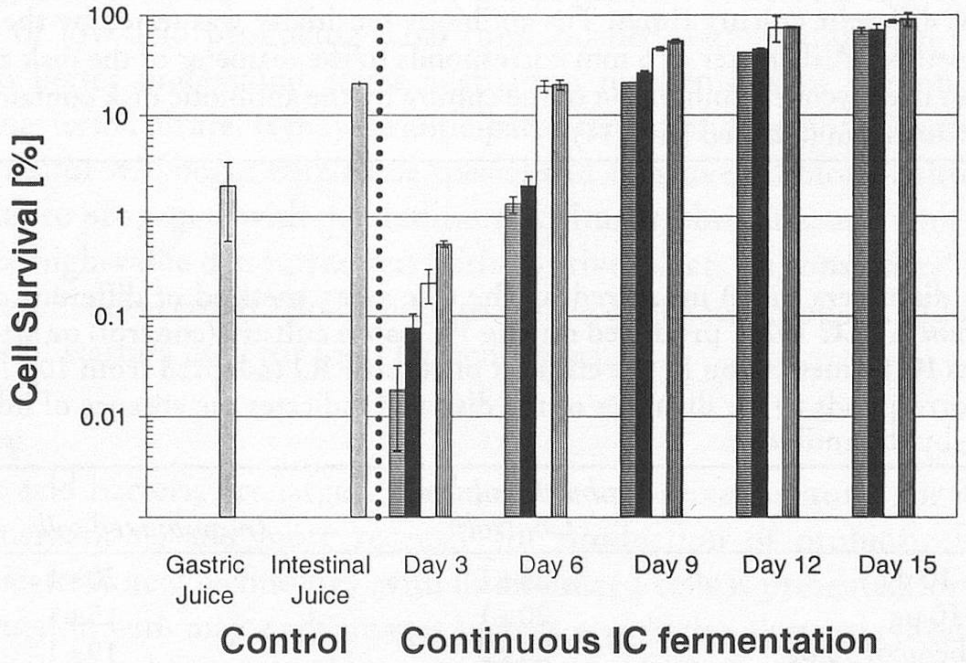

Figure 6 **Cell survival to simulated gastric and duodenal juices of *B. longum* from control (batch free-cell culture) and experimental (continuous two-stage IC fermentation) cultures, for reactors R1 and R2 and different culture times (adapted from 14) = ▤ R1 – gastric juice; ■ R1 – intestinal juice; □ R2 – gastric juice; ▤ R2 – intestinal juice**

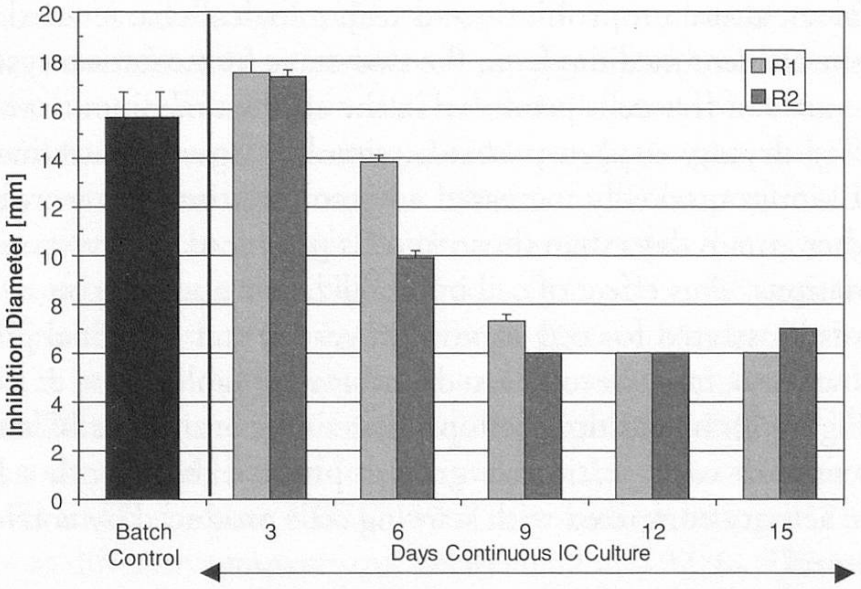

Figure 7 **Tolerance to kanamycin of *L. diacetylactis* cells produced during batch FC culture (control) or during continuous IC fermentation in reactors R1 and R2 at different culture times.** The antibiotic sensitivity was tested by the disc assay method. A diameter of 6 mm corresponds to the diameter of the disk and indicates the absence of inhibition of the culture by the antibiotic disk containing 30 mg kanamycin (adapted from 14)

Table 1
Inhibition diameters (mm) measured by the disc assay method of different antibiotics on *B. longum* ATCC 15707 produced during FC batch culture (control) or after 15 days continuous IC fermentation in the effluent of reactor R2 (adapted from 10). A diameter of 6 mm corresponds to the diameter of the disk and indicates the absence of inhibition of the culture by the antibiotic

Antibiotics	*Inhibition (mm)*[2] *Control*[1]	*Immobilized cells – 15 days*
Ampicillin 10 µg	25±1	20±1
Bacitracin 10 µg	20±1	15±1
Chloramphenicol 30 µg	26±2	19±1
Érythromycin 15 mg	28±2	24±1
Penicillin G 10 µg	21±1	16±1
Vancomycin 30 µg	20±1	0±0
Nisin Z 200 Ul/ml	11±0.3	7±0.2

A progressive reversibility of the acquired tolerance of *B. longum*, but not for *L. diacetylactis*, to antibiotics was shown during seven successive batch free-cell cultures initially inoculated with cells from the effluent of continuous IC fermentation (14). Our data indicate the potential to propagate and use in foods stress-adapted cells produced with the IC technology while keeping the acquired characteristics and are protected by patent (15).

Conclusions

Many advantages have been demonstrated for IC systems that can be applied to LAB and probiotic bacteria for the food and starter industries. Immobilized cell technology and continuous culture can be used to efficiently produce cultures (pure or mixed strains) with changed physiology and enhanced tolerance to various environmental stresses in a one step process and without the need for preconditioning treatments that may result in reduced cell activity and yield.

Specific effects of immobilisation on the physiology and technological characteristics of both entrapped and released cells have been recently shown. Application of this research could be particularly important for the production of probiotic bacteria, functional dairy products containing high concentrations of viable bacteria and bioingredients from LAB with enhanced technological and functional properties for use in foods and health products. In addition we recently showed that the immobilized cell technology allows to stably produce complex cultures composed of bacteria with different functional properties (bacteriocin, exopolysaccharide and aroma production) and that are not necessarily compatible (e.g. association of nisin-producing and nisin-sensitive bacteria), and to increase acidifying activity and acid tolerance of low acid producing LAB (unpublished data). Additional studies are needed to better understand stress adaptation mechanisms of immobilised cells during long-term culture. It may be anticipated that application of IC technology in the dairy sector will begin with these special and sensitive probiotic cultures which are difficult to propagate with the traditional culture techniques, and which are used to produce high-value dairy products with positive effects on consumers' health (5). Cell immobilization could also be used to enhance the technological and functional properties of starters used for food fermentations.

Summary

Lactic acid bacteria are largely used in pure and mixed cultures for traditional food fermentations, and more recently for production of probiotic foods and nutraceuticals. A new technology with immobilized cells is presented for food culture production with major advantages such as very high volumetric productivities, high biological stability over long-term cultures, high flexibility, and improved process control and product yields compared to traditional batch processes. Immobilized cell technology and long-term continuous culture have also been recently proposed to efficiently produce, in a one step process, cells with enhanced tolerance to environmental stresses. Application of this technology could be particularly promising for the production of probiotic bacteria, functional dairy products containing high concentrations of viable bacteria and probiotic preparations with important functional properties for use in foods and health.

Zusammenfassung

Milchsäurebakterien werden sowohl in reinen und als auch gemischten Kulturen zur Fermentierung von traditionellen Lebensmitteln häufig verwendet. In neuerer Zeit finden sie auch immer mehr Verwendung bei der Herstellung von probiotischen Lebensmitteln und Neutraceuticals. In dieser Arbeit wird eine neue Technologie mit immobilisierten Zellen für die Produktion von Lebensmittelkulturen vorgestellt. Diese hat gegenüber der traditionellen Herstellung mittels Batches den Vorteil einer sehr grossen volumetrischen Produktion, hoher biologischer Stabilität bei Langzeitkulturen, hoher Flexibilität und einer verbesserten Prozesskontrolle sowie grösserer Produkteerträge. Die Technologie der immobilisierten Zellen und die kontinuierlichen Langzeitkulturen wurden kürzlich auch zur effizienten Produktion, in einem einstufigen Prozess, von Zellen mit einer erhöhten Toleranz gegen Umweltstressfaktoren vorgeschlagen. Die Anwendung dieser Technologie könnte speziell für die Produktion von probiotischen Bakterien, von funktionellen Milchprodukten mit einem hohen Gehalt an lebenden Zellen und von probiotischen Lebensmitteln mit wichtigen funkionellen Eigenschaften für die Verwendung in Ernährung und Gesundheit interessant sein.

Résumé

Les bactéries lactiques sont largement utilisées en culture pure ou en mélange dans les fermentations alimentaires traditionnels et, plus récemment, dans la production d'aliments probiotiques et de nutraceutiques. Une nouvelle technologie avec cellules immobilisées est présentée pour la production de cultures alimentaires, avec plusieurs avantages importants comme une productivité volumétrique très élevée, une grande stabilité biologique lors des cultures à long terme, une haute flexibilité, un meilleur contrôle du procédé et des rendements plus élevés en comparaison avec les procédés classiques de culture en batch. La technologie des cellules immobilisées mise en jeu dans une culture en continu à long terme été récemment proposée pour produire efficacement, en une seule étape, des cellules a ayant une tolérance accrue aux stress environnementaux. L'application de cette recherche pourrait être particulièrement importante pour la production de bactéries probiotiques sensibles, de produits laitiers fonctionnels contenant des concentrations élevées en cellules viables et de préparations probiotiques ayant des propriétés fonctionnelles élevées pour leur utilisation dans les aliments et dans le domaine de la santé.

Key words

Cell immobilization, lactic acid bacteria, probiotics, continuous fermentation, cell physiology

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