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REVIEW PAPER

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STARTER CULTURES BIOTECHNOLOGY: THE PRODUCTION OF CONCENTRATED LACTIC CULTURES IN ALGINATE BEADS AND THEIR APPLICATIONS IN THE NUTRACEUTICAL AND FOOD INDUSTRIES

Lactic cultures are widely used in food fermentations and as probiotic supplements. Some strains are damaged by the various steps of fermentation, concentration and drying involved in the biotechnology process. Growing cells in alginate beads instead of free cells in a culture medium can answer some of these problems. In this review, the characteristics of the fermentation process based on growing microentrapped (ME) lactic cultures in alginate beads are presented. Potential benefits to nutraceutical and food industries of ME cultures are also presented.

Key words. Microencapsulation, Microentrapment, Lactobacillus, Bifidobacterium, Lactococcus, Probiotics, Nutraceuticals.

Food biotechnology has multiple facets. With respect to food processing, there are two main outputs of biotechnology: the production of ingredients and the production of fermented foods. Indeed, microbial cultures serve for the production of many ingredients such as organic acids (acetic, citric, lactic, fumaric), amino acids (glutamate), thickeners (xanthan and gellan gums), enzymes (invertase, lactase, lipases, proteases), flavours (diacetyl) and flavour enhancers (yeast extracts, nucleotides, soy sauce). In regards to fermented foods and beverages, two main groups of organisms are used: yeast and lactic acid bacteria (LAB). Yeasts particularly serve for the manufacture of wine, beer and bread. The LAB, on the other hand, are the most widely used bacteria in the food industry. They are involved in the fermentation of milk (yoghurt, cheese, sour cream, kefir, cultured butter), vegetables (sauerkraut, pickles, olives, kimchi), meats (dry sausages), cereals (sourdoughs), and even fruits (malo–lactic fermentation of wine). Therefore, the LAB are an economically important group of organisms. The original aim of the use of LAB was to preserve foods, and thus inhibit the growth of spoilage and pathogenic cultures. But there are novel applications of biotechnology in food processing: the addition of probiotic cultures. Probiotic cultures are defined as "defined, live microorganisms which, administered in adequate amounts, confer a beneficial physiological effect on the host" [1]. They are reputed to benefit humans by reducing problems linked to lactose intolerance and diarrhoea, as well as reducing the risks of colon cancer and various other intestinal disorders [2]. Initially found in fermented milks such as yoghurt or kefir, they are now increasingly added to non–dairy products which are fruit or cereal–based.

Although the dairy industry still propagates the LAB required for their fermentation processes at some levels, most food processing companies prefer to purchase their cultures from specialized suppliers and carry out the direct inoculation of their substrates with the concentrated LAB. There are, consequently, two industrial groups involved in food biotechnology with LAB: the producers of the cultures and the manufacturers of fermented foods.

There are excellent reviews on the various methods which can be used for bioencapsulation [3], and this manuscript will thus focus on one of them, alginate–type beads. The literature also abounds with names applied to gel–entrapped bacteria: encapsulated, microencapsulated, immobilized, entrapped and microentrapped (ME). The latter will be used throughout.

WHY PRODUCE CONCENTRATED LACTIC CULTURES IN ALGINATE BEADS?

Advantages for producers of cultures

Suppliers of concentrated LAB cultures typically carry out the biomass production step under pH control in 2000 to 10000 L vats, recover the cells by centrifugation or filtration, and market the concentrates in either frozen (cans or pellets) or freeze–dried (powder) forms. This process generates a certain number of stresses to the cells, which generate sub–lethal or lethal cell damages during the manufacturing process. As a result, viability losses are observed in the final stages of production. Examples of stressful conditions include:

- Oxygen stress due to agitation during pH control
- Oxygen and pressure stresses during centrifugation or filtration
- Membrane damages during freeze–drying
  - due to freezing itself
  - due to the drying step itself

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Unfortunately, there may be interactions (additive effect) between some of these detrimental effects.

Not all species have the same sensitivities to these detrimental production conditions. Probiotic bifidobacteria, for example, are much more sensitive to oxygen than the streptococci used in yoghurt manufacture. For these sensitive strains, immobilized cell technology (ICT) may be helpful in carrying out the production of biomass in alginate beads.

In addition, alginate beads can be air dried [4], which is a less expensive drying process than freeze–drying. Alginate beads can also be stored in low water activity solutions and sold fresh [5].

Applications in the nutraceuticals industry

A nutraceutical can be defined as a product isolated or purified from foods that is generally sold in medicinal forms not usually associated with food. A nutraceutical is demonstrated to have a physiological benefit or provide protection against chronic disease [6]. Nutraceuticals are typically marketed in the form of a powder, caplets or pills.

Many studies have shown that probiotic cultures which are ME in alginate beads show improved survival to acid in the gastric environment or to bile solutions. Modification of the alginate bead surface to presumably make it more hydrophobic [7], does not necessarily reduce mass transfer phenomena [8]. However, in many cases fresh beads were used, and this might not reflect what would occur when consumers would take the product in the dried form, which is the typical form of marketing of nutraceutical products. It is important to mention that, even though there are numerous examples where ME in alginate is beneficial (Table 1), there are also reports with negative data [24, 33]. The reasons for these discrepancies could be the following:

- Method of bead production or coating method [18]; coating the alginate bead with chitosan, for example, greatly improves survival to gastric solutions [11,12]
- Size of the bead particle [13, 15]
- Cell load [14].

Applications in the food industry

Probiotic cultures

Most applications to foods of lactic cultures ME in alginate beads are related to the addition of probiotic cultures for the development of functional foods. A functional food can be defined as a food product similar in appearance to, or may be, a conventional food, is consumed as part of a usual diet, and is demonstrated to have physiological benefits and/or reduce the risk of chronic disease beyond basic nutritional functions [6].

The most frequent application was the incorporation of probiotics to yogurt or fermented milks (Table 1). As was the case for nutraceuticals in the gastric environment, some data showing negative results heve been published [34,57]. The reasons for the discrepancies are not clear, but they might be due to the conditions of incubation with respect to oxygen content. Indeed, it was shown that the beneficial effect of ME on the survival of probiotics in yogurt principally occurred when the products were incubated under aerobic conditions [32].

A clear benefit of the ME of probiotics is survival when exposed to freezing during ice cream production (Table 1). This was influenced by bead size, and a critical aspect seemed to be the incorporation, inside the alginate beads, of a cryoprotectant such as glycerol [24]. These data suggest that the benefit of ME was linked to the creation of a microenvironment inside the alginate bead. Thus, during processing, the cells which are inside the alginate particle are in a chemical environment which is different from that in which the free cells are exposed to in the food matrix.

A list of other benefits during food processing or storage can be seen in Table 1.

Starter cultures

The benefits of ME in food processing with starter cultures are less numerous (Table 1). Generally speaking, ME in alginate tends to reduce the acidifying activity of the cultures [24,35,36] due to limitations in the mass transfer. However, there is one example (dry sausages) in which ME was beneficial [19]. Other benefits include protection against bacteriophages and

Table 1. Beneficial effects of the microentrapment of lactic cultures in alginate matrices in nutraceutical and food applications

Benefit	Product	Reference
Improved survival to exposure to gastric solutions	Nutraceutical	[7–15]
Improved survival to exposure to bile solutions	Nutraceutical	[8,10,12,14]
Cultures can be air–dried	Nutraceuticals	[16,17]
Improved stability during storage in dried form	Nutraceuticals	[9]
Improved acidification rate	Dry sausages	[19]
Improved survival to heating	MRS solutions	[20]
	Biscuits	[21]
	Powder	[22]
Improved survival to freezing	Ice cream	[23]
	Milk–based medium	[24]
	Cranberry juice	[21]
Protection against bacteriophages	Fermented milks	[22,25,26]
Protection against yeast contaminants	Fermented milks	[27]
Improved survival during storage	Yoghurt	[10,24,28–32]
	Mayonnaise	[58]
	Milk	[11,33]

increased survival during exposuere to heating (Table 1). Cultures can also be less sensitive to antimicrobial compounds in the food matrix [37].

PRODUCTION OF ALGINATE BEADS

One study suggests that alginate is better than whey protein gel beads [29] to protect cultures in subsequent applications. It is unknown if other gel matrixes made by ionic gelification (pectin, carragheenan) offer as good a protection as alginate, but alginate is the marix which is used the most. Thus the focus of this review is on alginate.

Three methods of producing alginate beads which carry LAB are most commonly used (Figure 1). The most popular is the alginate extrusion process in a calcium chloride solution, probably because it is so easy to carry out (Table 2). Some adaptations are seen in the literature. Starch [39], pectin and whey proteins [8] have been successfully mixed with alginate in order to improve the matrix in subsequent applications. Successful coating of the alginate beads can also be carried out by dipping them into chitosan [10,28,40] or poly–L–lysine [10, 41] solutions. Other coating or bead modification methods for alginate have been attempted by succinilation [7] or covalent bonding, but important losses in viability have resulted in some instances [42,43].

The scale up of bead production is possible with units having multiple droplet–producing nozzles (Figure 2)

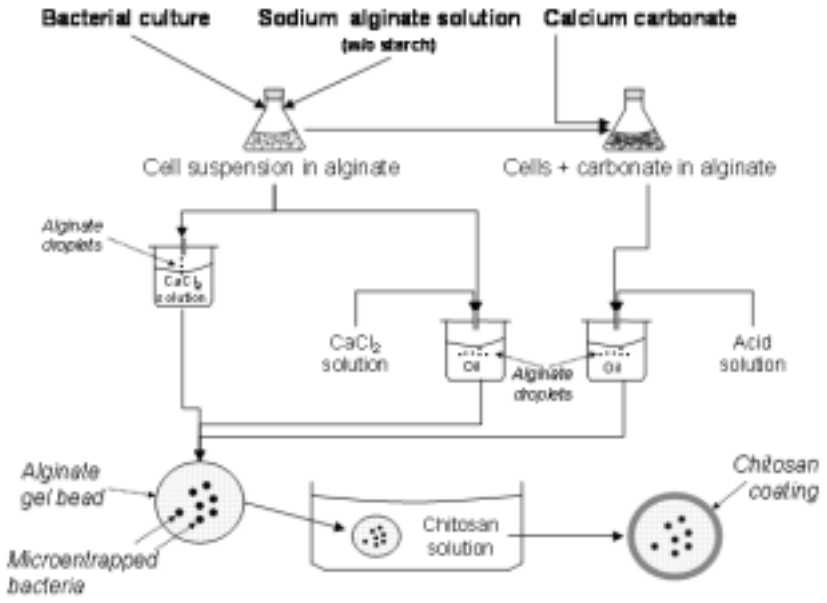

Figure 1. Some techniques for the production of alginate beads (w/o starch), as well as the coating procedure with chitosan

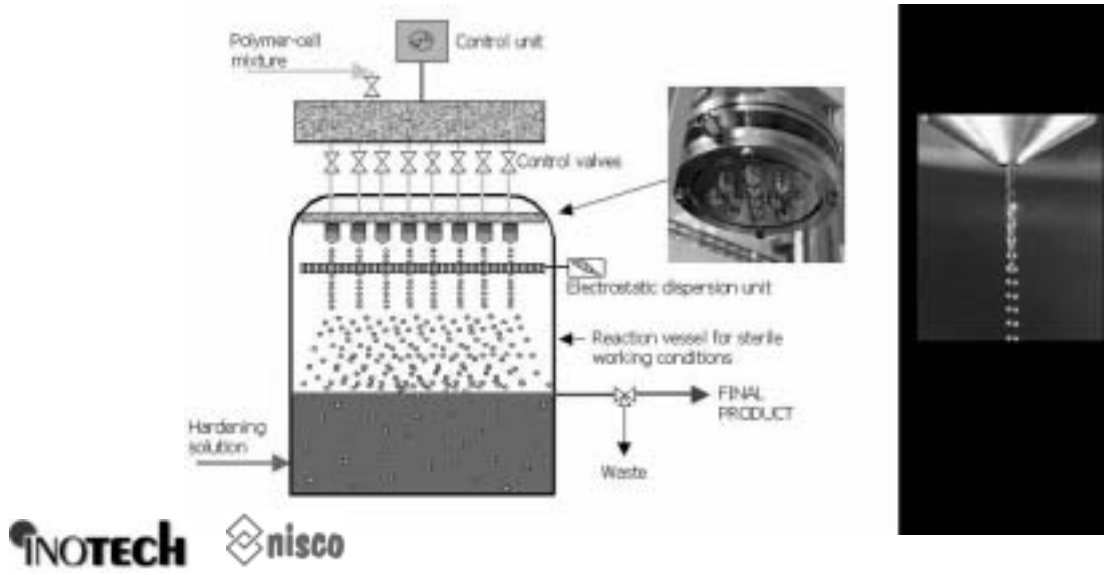

Figure 2. Large scale production of alginate beads by extrusion [44]

Table 2. Positive and negative features of extrusion and emulsion techniques [38].

	Extrusion	Emulsion
Technological feasibility	Difficult to scale–up	Easy to scale–up
Cost	Low	High
Simplicity	High	Low
Survival of microorganism	80–95%	80–95%
Size of bead	2–5 mm	25 μm – 2 mm

and this can be done in a continuous fashion [45]. Other methods have also been proposed on a larger scale. Begin et al. [46] have devised a rotative atomization unit to prepare beads on a larger scale, but the method is a function of the vat diameter required to accommodate the spread of the droplets ejected from the disk. A vortex–bowl unit was designed to address this problem, but variability in bead size was encountered [47]. A jet cutting unit has also been described [48].

BIOMASS PRODUCTION IN ALGINATE BEADS

Principles

Alginate beads were prepared by mixing a fresh culture with alginate solution and subsequently entrapping the cells in the gel. Since the alginate solution is mixed with the culture in ratios varying from 5:1 to 1:1, the culture is diluted very little. Therefore, populations in the beads at the beginning of the fermentation are between 10^8 and 10^9 CFU/g. The beads are then added to the growth medium. The inoculation levels range between 5 and 25% (v/v), typically at 10%. Theoretically, the nutrients in the medium diffuse inside the bead and support the growth of the microentrapped bacteria inside the gel (Figure 3). Lactic acid diffuses outside the bead and is neutralised in the broth thanks to the pH control components. Obviously, the pH inside the bead is slightly lower than that in the broth. In an ideal situation, growth only occurs inside the bead and the biomass that would be dispersed throughout the broth in a free cell fermentation is limited to the gel bead. Thus, if the medium was inoculated at 10% with beads, the population inside the bead would be 10 times more concentrated than it would be in a broth with free cells, and 20 times higher if the medium were inoculated at 5% (v/v). As data on "population yields" will show, this ideal situation rarely occurs.

Since the beads are rather large, generally between 1 and 3 mm, they can easily be recovered by sifting of the medium. Low g centrifugation would also be possible. This technology, therefore, enables the production of concentrated cultures without the need to centrifuge the fermented broths at high g levels or to use micro– or ultrafiltration processes.

An important aspect of the process is the requirement of free Ca^{2+} in the broth so as to maintain the integrity of the beads. As lactic acid accumulates, it generates Ca^{2+} release from the bead and the gel softens. Therefore, the growth medium must contain enough Ca^{2+} to maintain bead integrity. The presence of $CaCO_3$ in the fermentation medium helped maintain the firmness of the alginate beads despite the increased production of lactic acid during fermentation or the use of a phosphated medium [49].

Population yields

Under continuous culture, very high bacterial densities (between 10^{10} and 10^{11} CFU/g) can be reached in calcium alginate beads [50]. It was thus

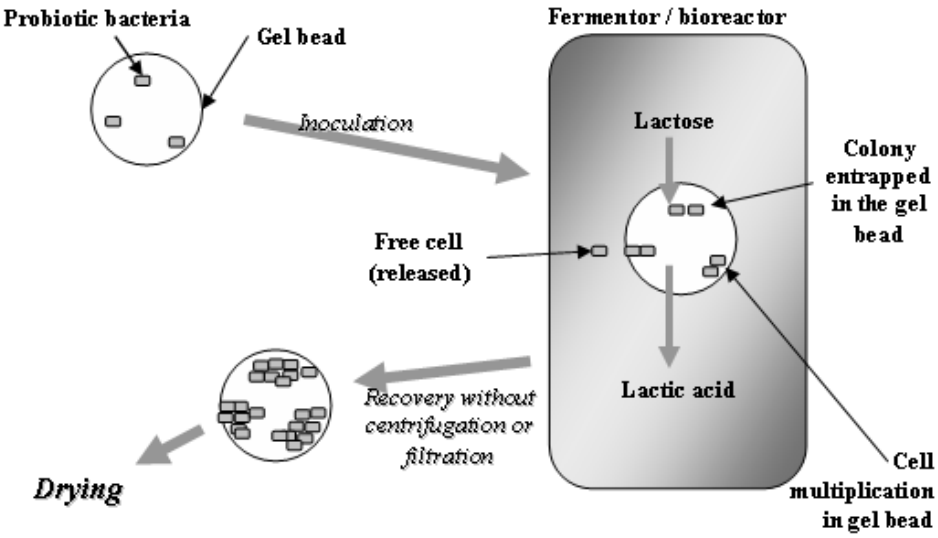

Figure 3. Principles of biomass production of LAB in alginate beads

attempted to obtain such high densities in beads during a single batch fermentation, carried out under pH control.

In the case of with *Lc. lactis* the total cell populations were much higher in the classical free–cell fermentation. The bead–entrapped population represented only 25% of that obtained in free–cell fermentation [49,51] and free cells represented about half of the population in the bioreactor [51].

The same phenomenon appeared with thermophilic cultures. With free cells, in a fermentation carried out with a mixed culture of *Streptococcus thermophilus* and *Lactobacillus delbrueckii ssp. bulgaricus*, which is typically used in yoghurt or mozzarella manufacture, continuous neutralization with NaOH or NH_4OH at pH 5.8 more than doubled the populations: streptococci counts increased from $8x10^8$ to $2x10^9$ CFU/mL, while that of the lactobacilli increased from $3x10^8$ to $8x10^8$ CFU/mL [52]. Although immobilized cell technology allowed the production of concentrated cultures in the alginate gels five times higher than with classical free cell suspensions, the overall cell count obtained was 25% lower than in classical free cell systems [52]. There was considerable cell release, and only 40% of the overall biomass at 46°C was in the beads.

We examined the situation with other cultures to determine if low biomass yields were inherent to ICT. Fermentations carried out with *Lactobacillus rhamnosus* RW9595M also resulted in approximately half the yield in biomass in the ICT bioreactor, confirming previous data with *Lb. rhamnosus* [39]. However, *Lactobacillus plantarum* gave somewhat better results, with ICT yields being 70% of that in free cells. The best results were obtained with *Leuconostoc mesenteroides* in which the biomass yields in alginate beads were the same as those in free cells. Therefore, lower biomass yields with ICT are generally encountered, but should not be automatically assumed because there are instances where the technology is entirely satisfactory. Overall data suggest that the effect of the strain seems important in the yield, but they also suggest that mesophilic cultures might be better adapted to the technology. This being said, the fermentation temperature can be used to reduce cell release, and this aspect will be examined further.

The very high populations which are attained in alginate beads used in continuous inoculation systems [50], pointed to the possibility that greater yields would be obtained by carrying out multiple batch fermentations. Indeed, removing a fermented medium, adding a fresh broth and re–using the same beads for successive fermentations enables further increases in bead populations, and this was demonstrated for *Bifidobacterium longum* (Figure 4). Even though these data were obtained on K–carrageenan locust bean gum beads [54], a similar phenomenon was noted with

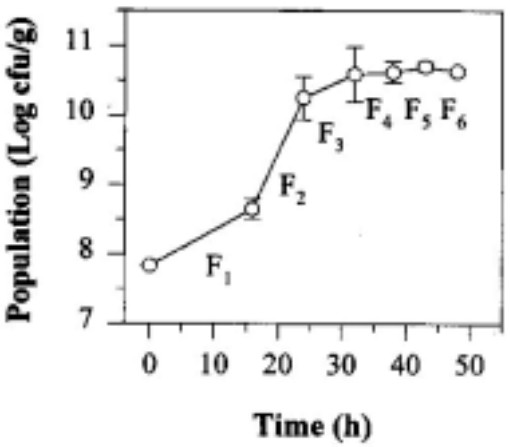

Figure 4. Growth of Bifidobacterium longum ATCC 15707 in K–carrageenan–locust bean gum beads during six successive fermentations (F_1 to F_6) using the same beads [54]

Lactococcus lactis in alginate [51]. However, there are limits to this strategy. The second and third fermentations must be very short [51], and growth improvements are not notable after the third fermentation (Figure 4).

With bifidobacteria, the addition of calcium carbonate to the alginate bead was beneficial to growth [55]. It remains to be seen if such a benefit can extend to other cultures.

With respect to yields, a caution might need to be given. The literature suggests that total CFU values in a bioreactor were generally lower when the cultures were produced in beads. However, it has been reported that lactobacilli can have longer chain lengths when growing in alginate beads [53]. Not all teams incorporate a chain–breaking step at the beginning of dilutions carried out for CFU analyses. It is unknown if growth in alginate beads systematically generates longer chains for the lactobacilli. If this is the case, then CFU counts underestimate the biomass, and the lower yields reported might not be as extensive as first thought.

Effect of pH

In a mixed culture of *Streptococcus thermophilus* and *Lactobacillus delbrueckii ssp. bulgaricus*, growth in the alginate beads was significantly affected by time, pH and medium. Moreover, the effect of time and media varied depending on the studied pH. Even the triple interaction between medium, pH and time was highly significant with the Lactobacillus. Under optimum conditions for the production of the lactobacilli (whey + meat extract, pH 6.1) the *S. thermophilus* population reached only 2 ¤ 10^9 CFU/g, which was inferior to that obtained in the control [52]. These conditions, however, did enable the production of a culture having a 1:1 ratio. Under the optimum growth conditions for the streptococci (milk, pH 5.9), the cocci : rod ratio was 10:1 [52]. Therefore, when mixed cultures are grown, the pH

not only affects growth rates and yields, but also strain ratios. With *Lc. lactis*, in most media growth in the beads was not affected by the pH of the surrounding medium when the latter was between 5.6 and 6.8. [49].

When producing lactic cultures, bead swelling, dissolution or contraction occurs as a function of pH. Thus, the amount of alginate beads recovered at the end of fermentations is significantly influenced by the pH of the medium [52]. Between pH values of 5.2 and 6.1, there was a linear increase in recovered bead mass and pH [52].

As a rule, higher populations were obtained when pH control was carried out. However, even if the acid was neutralised, growth stopped due to the accumulation of inhibitory metabolites. Typically, lactate levels between 3 and 6% will significantly reduce growth rate, but Na and NH_4 ions from the base solutions can also be problematic [55].

Reducing free cell levels

Because cell release from the beads significantly reduces biomass yields, finding ways to limit cell release is critical from an economic standpoint.

With thermophilic cultures, an attempt was made to proportionally increase the population of the beads by reducing the temperature of incubation. Since lowering of the temperature has a more marked effect on the growth rate than on the diffusion of the metabolites, it was hoped that a slower growth rate would reduce substrate utilization at the surface of the beads, and enable a greater part of the substrates to reach the inner bead. Fermentations carried out at 24°C for 18 h allowed an increase in the bead cell count which then constituted 60% of the population in the system (Table 3). However, lowering of the incubation temperature was detrimental to the growth of the lactobacilli, and the population obtained was essentially that of *S. thermophilus* Y24.

A second method used to reduce cell release is incubating at high pH levels. With thermophilic cultures, in a pH range between 5.2 and 6.1, the highest free cell levels were generally obtained at pH and media conditions that also favoured the highest levels of entrapped cells [52]. Therefore, pH values outside needed to be examined for the purpose of reduction of the cell release. Since the pH inside the bead is lower than at the surface, adjusting the pH of the broth above the optimum pH for growth, favours growth at the center of the bead rather than at the surface. Thus, change in operating pH from 6.5 to 9.25 initially reduced the ratio of the rates of cell release to lactate production by almost a factor of 10^5 [56]. Compared with fermentations at pH 6.5, growth at pH 9.25 also increased the final internal bead biomass concentration by a factor of 5 [56].

Other means to reduce cell release include coating the alginate bead with chitosan or killing the cells at the surface [41].

Table 3. Effect of incubation temperature on total populations (x 10^{11} CFU per 150 mL) in a bioreactor obtained in the control (free cell) and in an immobilized cell system with a mixed culture of Streptococcus thermophilus (St) and Lactobacillus delbrueckii ssp. bulgaricus (L.b), under pH control [52].

Fermentation	Beads		Medium		Total
	S.t.	L.b.	S.t.	L.b.	
Free cell control (46°C)	–	–	16.5	5.4	21.9
Alginate beads (46°C)	5.5	0.3	9.2	0.3	15.3
Alginate beads (24°C)	8.0	< 0.1	5.4	< 0.1	13.4

CONCLUSION

Although alginate beads can be produced rather easily, the microentrapment of lactic cultures in such particles for the purpose of producing a biomass adds a step in the culture production process. There must, therefore, be a good reason to carry out this technological process. Many have been proposed, with respect to both the producers of starters and probiotic cultures or for food manufacturers as such. But lately, food products have been marketed in which probiotic cultures are marketed in particles, and consumers can clearly identify the food component which contains the beneficial cultures. Therefore, in addition to the technological benefits of ME, it can be predicted that the biotechnology process of production of lactic cultures in alginate particles will be useful to companies from a marketing standpoint.

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IZVOD

BIOTEHNOLOGIJA STARTER KULTURA: PROIZVODNJA KONCENTROVANIH KULTURA BAKTERIJA MLEČNE KISELINE U ALGINATNIM ČESTICAMA I NJIHOVA PRIMENA U INDUSTRIJI NUTRACEUTIKA I PREHRAMBENOJ INDUSTRIJI

(Pregledni rad)

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Kulture bekterija mlečne kiseline su u širokoj primeni kako u fermentacionim procesima u prehrambenoj industriji, tako i kao probiotski saplementi. Međutim, neki sojevi ovih bakterija mogu biti oštećeni tokom različitih faza biotehnoloskih procesa, kao što su pojedini stadijumi fermentacije, koncentrisanja i sušenja. Neki od ovih problema mogu biti rešeni ukoliko se umesto ćelija suspendovanih u hranljivom medijumu primene ćelije imobilisane u alginatnim česticama. U ovom preglednom radu su prikazane karakteristike fermentacionih procesa baziranih na mikroimobilsanim rastućim ćelijama bakterija mlečne kiseline u alginatnom nosaču. Takođe su prikazane moguće koristi primene mikroimobilisanih kultura u industriji nutraceutika i prehrambenoj industriji.

Ključne reči: Mikroinkapsulacija, Mikroimobilizacija, *Lactobacillus, Bifidobacterium, Lactococcus*, Probiotici, Nutraceutici.