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ORIGINAL PAPER

Preparation of lactic acid bacteria-enclosing alginate beads in emulsion system: effect of preparation parameters on bead characteristics

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Abstract The immobilization of plant growth-promoting bacteria in biodegradable polymeric supports is effective in providing them with a suitable microenvironment for increased survival, compared to free bacteria, in agricultural land. In this study, we optimized the preparation parameters for bacteria-enclosing calcium alginate gel beads with a view to large-scale production. An emulsion system was used and the optimization was based on alginate bead recovery and entrapment efficiency of viable bacteria. Lactic acid bacteria were used as a plant growth-promoting bacteria. The optimized conditions were as follows: the concentration of calcium chloride in the aqueous phases was 1.1% (w/v), the volume ratio of alginate solution to total aqueous phases was 0.93, and the agitation time was 0.5–1.0 h. The mean diameter of the beads could be controlled (approximately from 100 to 300 μm) by varying the agitation rate.

Keywords Microencapsulation · Lactic acid bacterium · Soil bioamendment · Alginate bead · Emulsion system · Large-scale production

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Introduction

Chemical fertilizers and pesticides are essential for maintaining large-scale and stable production of agricultural products and reducing labor requirements [1]. However, concerns about environmental sustainability have resulted in increased interest in limiting the use of the chemicals [2]. Soil bioamendment (SBA), in which bacteria produce and release various metabolites that are beneficial for plant growth and health, offers a promising alternative to minimizing usage and reduction of the deleterious effects of these chemicals. The application of SBA to agricultural soils has been shown to enhance crop yield [3]. Plant growth promotion by the bacteria is achieved through phytostimulation, biofertilization, or biocontrol of plant pathogens [4, 5].

Plant response to inoculated SBA varies according to the type of bacteria used, the plant species, inoculation density, soil type, and various environmental conditions. The key obstacle is the unpredictability of the environment and the heterogeneous nature of agricultural soil [6]. Free bacteria may be unable to locate a niche in the soil to ensure their survival among competitors and predators. This results in decreased bacterial density and failure to elicit the intended plant response [6]. Immobilization of the bacteria in biodegradable microcapsules provides a more suitable environment for their survival. The microcapsules provide temporary protection of the immobilized bacteria from soil environment, competitors and predators. They also allow gradual release of the bacteria in a degraded form, which is an effective way to establish the bacteria within the targeted soil. Another advantage of such microcapsules is the fact that they can be handled more easily than free bacteria.

Polymeric hydrogels have been widely used as materials for these microcapsules [7–14]. Calcium alginate hydrogel beads are one of the most frequently utilized hydrogel materials due to their innocuousness and ease of gel formation [15–17]. Once liquid sodium alginate solutions come in contact with divalent cations such as calcium ions, they are immediately transformed into a gel due to binding between the cations and guluronic acid blocks in alginate polymer. Two widely used methods for the immobilization of bacteria in alginate beads are extrusion and emulsification [15]. Extrusion, which was the earliest developed method for preparing alginate beads [18], simply involves preparing an alginate solution, adding bacteria to it, and extruding the cell suspension through a syringe needle in the form of droplets to free-fall into a calcium chloride solution. In the emulsion method, an alginate solution containing bacteria is dispersed in a large volume of an oil phase such as vegetable oil [15, 16], and calcium chloride solution is then dispersed in the emulsion. Gelation of the alginate solution droplets in the emulsion is achieved by contact between the polymer solution and the calcium chloride solution droplets. The extrusion method is time-consuming to obtain a large amount of the alginate beads. On the other hands, the emulsion method has a potential for large-scale production of the beads in shorter time than extrusion one. The large-scale production is essential for commercial application of the beads as SBA.

Lactic acid bacteria (LAB), which produce lactic acid as a metabolite, are expected to be useful for improving the condition of agricultural soil. Lactic acid has a potential to control plant pathogens in soil (Hiramatsu K., Japanese patent 2005-230000, 2005). Although there have been some reports on the encapsulation of LAB in alginate beads using an emulsion system, no researchers have carried out

optimization of the preparation parameters from the view point of large-scale production and cost saving [15–17].

The purpose of the current study was to optimize the preparation parameters in emulsion system for large-scale production of the LAB-enclosing alginate beads and cost saving for the production. Specifically, we first investigated optimum concentration of calcium chloride in the system, which enabled formation of alginate beads and did not induce cell damage. Then, we examined the volume ratio of alginate solution to calcium chloride solution, and agitation time on alginate bead recovery and entrapment efficiency of viable LAB.

Experimental

Materials and microorganism

Lactobacillus delbrueckii subsp. *bulgaricus* NBRC 13953 was obtained from the NITE Biological Resource Center (NBRC, Chiba, Japan). The strain was maintained in 803 medium (5 g of glucose, 5 g of yeast extract, 5 g of polypepton, 2 g of lactose, 1 g of $MgSO_4 \cdot 7H_2O$, and 0.5 g of polyoxyethylene (20) sorbitan monolaulate in 1 L distilled water (pH 6.5–6.8) at 37 °C. Sodium alginate (80–120 mPa s), calcium chloride and sorbitan monooleate were purchased from Wako Pure Chemical Co. (Osaka, Japan).

Preparation of LAB-enclosing alginate beads

The preparation apparatus was a round-bottomed glass-jacketed vessel (volume: 1,000 cm^3) equipped with a mechanical stirrer. The stirrer was fitted with a 77 mm crescent Teflon-coated blade. Sodium alginate was dissolved in 803 medium containing a suspension of LAB at a concentration of 2.0% (w/v) (alginate aqueous phase, W_{alg}). The viable cell number of LAB in W_{alg} was determined to be the number of colony forming units (cfu) on an agar medium (803 medium containing 1.5% agar, 1.0–2.0 $\times$ 10^8 cfu/mL). The suspension was dispersed in canola oil with 0.5 wt% dissolved sorbitan monooleate (oil phase, O) under agitation at 22 °C for 30 min. Subsequently, calcium chloride solution (W_{cal}) was added to the emulsion at a rate of 10 mL/min under agitation. After agitation for several hours, the mixture was filtered and beads were collected. The agitation rate was fixed during the bead preparation process. The volume of the oil phase and the total volume of the aqueous phases ($W_{alg} + W_{cal}$) were fixed at 200 and 150 mL, respectively. The experimental conditions are shown in Table 1.

Table 1 Experimental condition

$CaCl_2$ concentration (amount of $CaCl_2$/total volume of aqueous phases)	1.1, 3.3, 5.0, 10.0% (w/v)
Volume ratio of W_{alg} to total aqueous phases [$W_{alg}/(W_{alg} + W_{cal})$]	0.53, 0.67, 0.80, 0.93
Agitation time after addition of W_{cal}	0.5, 1.0, 3.0, 6.0 h
Agitation rate	70, 100, 150, 200 rpm

Determination of entrapment efficiency of viable LAB

A portion (1–2 g) of collected beads was transferred into 50 mL aqueous solution with 55 mM dissolved trisodium citrate (pH 7.0) and gently shaken for 30 min to liquefy the beads. The viable cell number in the solution was determined using an agar medium as described above. The entrapment efficiency of viable LAB was calculated on the basis of the viable cell number.

Determination of alginate bead recovery

Alginate bead recovery was determined by comparing the weights of the dried alginate beads and the sodium alginate powder used for bead preparation. A portion (9–12 g) of alginate beads prepared via the emulsion method was carefully washed with distilled water to remove medium components from the beads and further washed with hexane to eliminate canola oil. The beads were then immersed in 1.1% (w/v) calcium chloride solution, followed by distilled water and dried under vacuum. Alginate bead recovery was determined on the basis of the weight of the dried beads.

Results and discussion

Effect of calcium chloride concentration

Alginate solution droplets (W_{alg}) in this emulsion system are gelled by contact with calcium chloride solution droplets (W_{cal}). The concentration of calcium chloride in W_{cal} has an important influence on the characteristics of the resulting alginate beads. Therefore, we first examined the effect of the concentration on alginate bead recovery and the entrapment efficiency of viable LAB.

The volume ratio of W_{alg} to the total aqueous phases, agitation time after addition of W_{cal} and agitation rate were fixed at 0.67, 3.0 h and 100 rpm, respectively. The concentration of calcium chloride in W_{cal} was varied from 3.3% (w/v) to 30.0% so that the concentration of calcium chloride based on the total volume of the aqueous phases (amount of calcium chloride/total volume of aqueous phases) varied from 1.1% (w/v) to 10.0%. The mean diameters of the alginate beads were approximately 300–400 μm under all preparation conditions (data not shown). The concentration of calcium chloride was found to have scarcely any influence on alginate bead recovery (Fig. 1), with values of approximately 90% obtained for all concentrations. This means that a concentration of 1.1% (w/v) is sufficient for the formation of alginate gel beads. The entrapment efficiency of viable LAB was found to decrease at concentrations of more than 5.0% (w/v), with a value of less than 1.0% at a concentration of 10.0% (w/v). This may indicate damage of LAB due to osmotic stress. Based on these results, we adopted a concentration of 1.1% (w/v) in subsequent experiments.

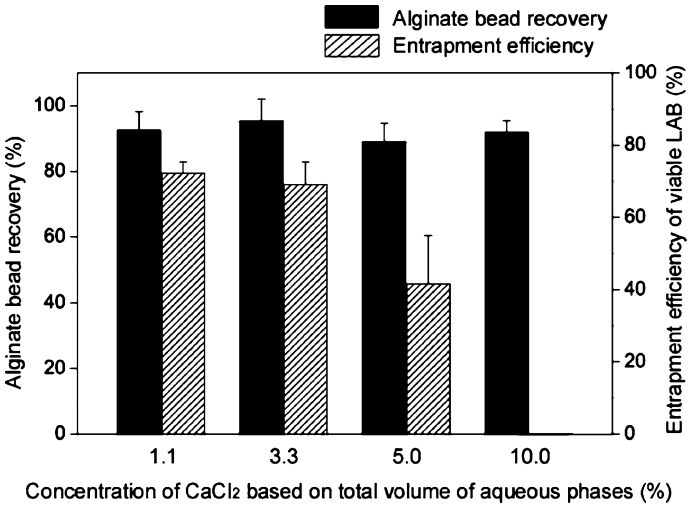

Fig. 1 Effect of concentration of calcium chloride on alginate bead recovery and entrapment efficiency

Effect of volume ratio

From the viewpoint of large-scale production of LAB-enclosing alginate beads for SBA, it should prove useful to increase the volume ratio of W_{alg} to the total aqueous phases ($W_{alg} + W_{cal}$). Therefore, we examined the effect of the volume ratio on alginate bead recovery and entrapment efficiency of viable LAB.

The volume ratio was varied from 0.53 to 0.93, with the calcium chloride concentration, agitation time after addition of W_{cal} and agitation rate fixed at 1.1% (w/v), 3.0 h and 100 rpm, respectively. The mean diameters of the alginate beads were approximately 250–350 μm under all preparation conditions (data not shown). The volume ratio was found to have little influence on alginate bead recovery and entrapment efficiency (Fig. 2). Thus, we succeeded in increasing the amount of obtained LAB-enclosing alginate beads by increasing the volume ratio without damaging enclosed LAB (the amount of obtained alginate beads at the volume ratio of 0.93 is approximately 1.4 times larger than that at the ratio of 0.67 on the basis of dry weight of the beads).

In the previous experiment, we showed that a high calcium chloride concentration in the aqueous phases resulted in cell damage. In this experiment, we initially expected that the increase in the calcium chloride concentration in W_{cal} associated with the increase in the volume ratio would cause cell damage because LAB would be exposed to a high concentration of calcium chloride for some time after the addition of W_{cal} [calcium chloride concentration in W_{cal} at a volume ratio of 0.93: 16.7% (w/v)]. However, cell damage was not observed. These results demonstrate that this emulsion system is useful for large-scale production of LAB-enclosing alginate beads. In subsequent experiments, we adopted a volume ratio of 0.93 and a calcium chloride concentration of 1.1% (w/v).

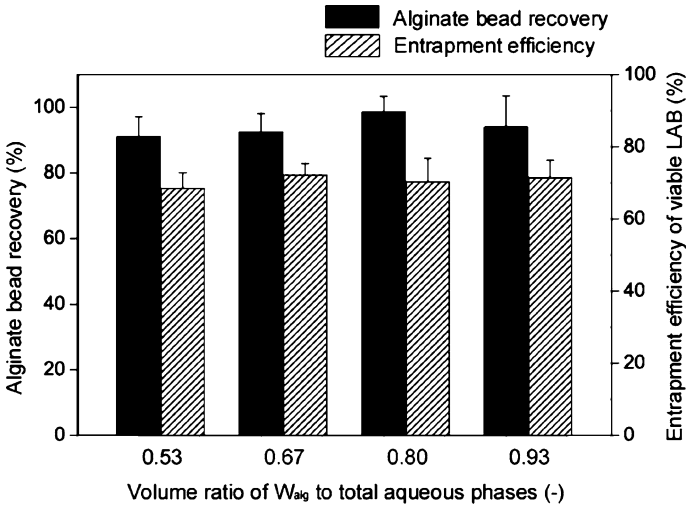

Fig. 2 Effect of volume ratio of W_{alg} to total aqueous phases on alginate bead recovery and entrapment efficiency

Effect of agitation time

Because reduction of the agitation time results in cost savings, we investigated the effect of agitation time after addition of W_{cal} to the W_{alg}/O emulsion on alginate bead recovery and entrapment efficiency of viable LAB.

The agitation time was varied from 0.5 to 6 h, with the calcium chloride concentration, volume ratio of W_{alg} to total aqueous phases and agitation rate fixed at 1.1% (w/v), 0.93 and 100 rpm, respectively. The mean diameters of the alginate beads were 250–350 μm under all preparation conditions (data not shown). As shown in Fig. 3, the agitation time had little influence on alginate bead recovery and entrapment efficiency, which means that a time of 0.5–1.0 h is sufficient for the formation of alginate beads. In subsequent experiments, we adopted an agitation time of 1.0 h, a volume ratio of 0.93 and a calcium chloride concentration of 1.1% (w/v).

Effect of agitation rate

In this experiment, we examined the effect of the agitation rate on bead size, alginate bead recovery and entrapment efficiency of viable LAB. As described in the Introduction, alginate beads enclosing plant growth-promoting bacteria allow gradual release of the bacteria in a degraded form, which is an effective way to establish the bacteria within the targeted soil. One of the factors that influences the degradation rate of the beads is their size, which depends on the agitation rate. Therefore, information on the relationship between the agitation rate and the size of the resulting beads is essential for controlling the bead size.

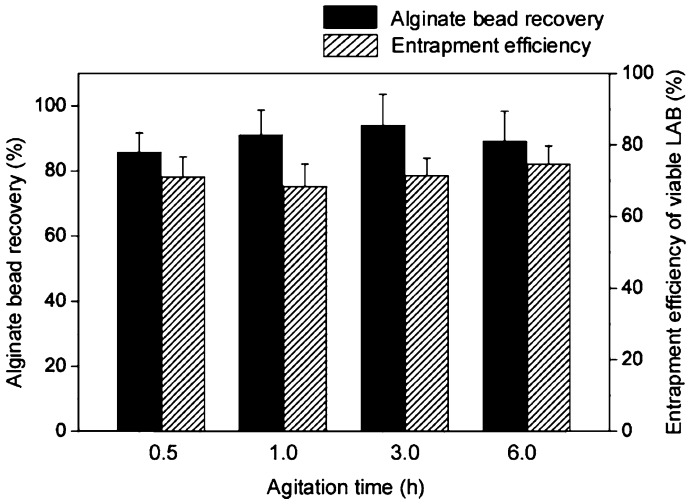

Fig. 3 Effect of agitation time on alginate bead recovery and entrapment efficiency

The agitation rate was varied from 70 to 200 rpm, with the calcium chloride concentration, volume ratio of W_{alg} to total aqueous phases and agitation time fixed at 1.1% (w/v), 0.93 and 1.0 h, respectively. Figure 4 shows the relationship between agitation rate and bead diameter, and Fig. 5 shows images of the resulting beads. The diameter of the alginate beads at 70 rpm was 309 ± 163 μm (mean ± SD). The diameter decreased as the agitation rate increased; the value at a rate of 200 rpm was 116 ± 52 μm. The agitation rate had little influence on alginate bead recovery or the entrapment efficiency of LAB (Fig. 6).

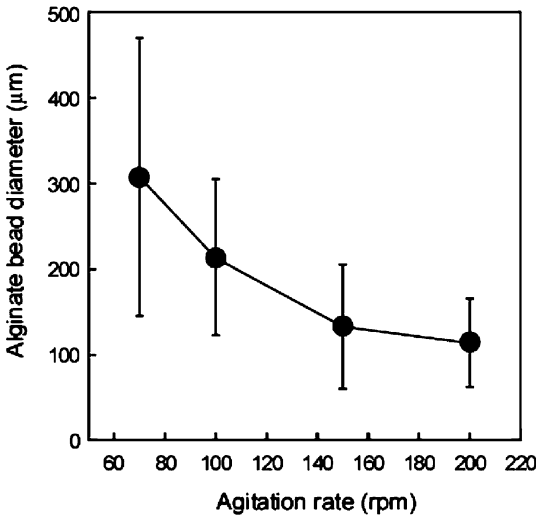

Fig. 4 Relationship between agitation rate and bead diameter

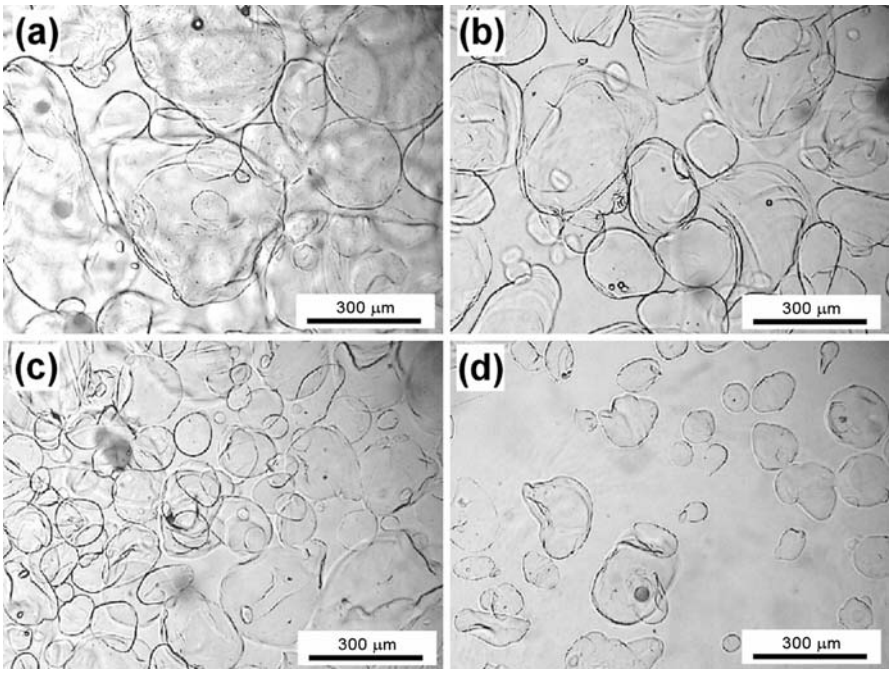

Fig. 5 Alginate beads prepared with different agitation rates: **a** 70 rpm, **b** 100 rpm, **c** 150 rpm, and **d** 200 rpm

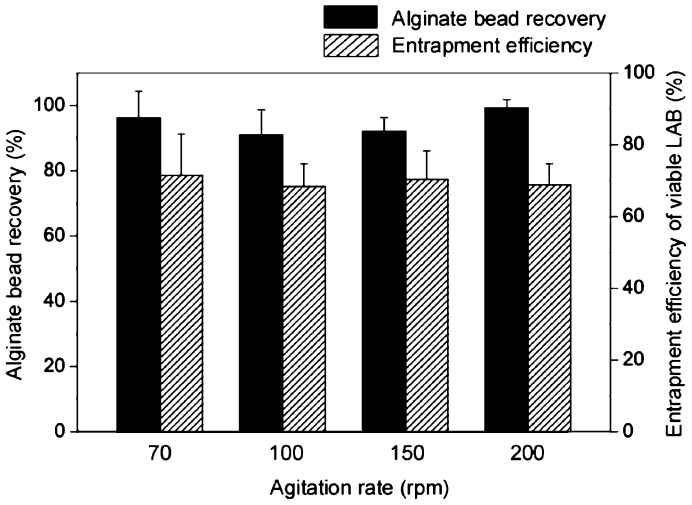

Fig. 6 Effect of agitation rate on alginate bead recovery and entrapment efficiency

Conclusion

In conclusion, we optimized the preparation parameters for LAB-enclosing alginate gel beads in an emulsion system with a view to large-scale production. The optimized concentration of calcium chloride in the aqueous phases, volume ratio of W_{alg} to total aqueous phases and agitation time were 1.1% (w/v), 0.93 and 0.5–1.0 h, respectively. The mean diameter of the beads was controlled between 100 and 300 μm (approximately) by varying the agitation rate.

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References

1. Shiomori K, Taniguchi J, Kiyoyama S, Kawano Y, Hatate Y (2004) Preparation and release characteristics of biodegradable microcapsules encapsulating activated carbon impregnated with pesticide using the solvent evaporation method. J Chem Eng Jpn 37:357–364
2. Greene LC, Meyers PA, Springer JT, Banks PA (1992) Biological evaluation of pesticides released from temperature-responsive microcapsules. J Agric Food Chem 40:2274–2278
3. Young CC, Rekha PD, Lai WA, Arun AB (2006) Encapsulation of plant growth-promoting bacteria in alginate beads enriched with humic acid. Biotechnol Bioeng 95:76–83
4. Bloemberg GV, Lugtenberg BJJ (2001) Molecular basis of plant growth promotion and biocontrol by rhizobacteria. Curr Opin Plant Biol 4:343–350
5. Bashan Y (1998) Inoculants of plant growth-promoting bacteria for use in agriculture. Biotechnol Adv 16:729–770
6. Vanelsas JD, Trevors JT, Jain D, Wolters AC, Heijnen CE, Vanoverbeek LS (1992) Survival of, and root colonization by, alginate-encapsulated pseudomonas-fluorescens cells following introduction into soil. Biol Fertil Soils 14:14–22
7. Stormo KE, Crawford RL (1992) Preparation of encapsulated microbial-cells for environmental applications. Appl Environ Microbiol 58:727–730
8. Trevors JT (1991) Respiratory activity of alginate-encapsulated pseudomonas-fluorescens cells introduced into soil. Appl Microbiol Biotechnol 35:416–419
9. Qi WT, Yu WT, Xie YB, Ma XJ (2005) Optimization of *Saccharomyces cerevisiae* culture in alginate–chitosan–alginate microcapsule. Biochem Eng J 25:151–157
10. Wang GJ, Chu LY, Chen WM, Zhou MY (2005) A porous microcapsule membrane with straight pores for the immobilization of microbial cells. J Membr Sci 252:279–284
11. Chen YM, Lin TF, Huang C, Lin JC, Hsieh FM (2007) Degradation of phenol and TCE using suspended and chitosan-bead immobilized *Pseudomonas putida*. J Hazard Mater 148:660–670
12. Yoshida M, Mardriyati E, Tenokuchi D, Uemura Y, Kawano Y, Hatate Y (2003) Structural control of core/shell polystyrene microcapsule-immobilized microbial cells and their application to polymeric microbioreactors. J Appl Polym Sci 89:1966–1975
13. Takei T, Yoshida M, Hatate Y, Shiomori K, Kiyoyama S (2008) Lactic acid bacteria-enclosing poly(ε-caprolactone) microcapsules as soil bio-amendment. J Biosci Bioeng 106:268–272
14. Kakizono K, Yoshida M, Hatate Y, Shiomori K, Kiyoyama S (2005) Biodegradable microcapsule immobilized bovine serum albumin by solvent evaporation of W/O/W emulsion. ITE Lett Batter New Technol Med 6:574–580
15. Homayouni A, Ehsani MR, Azizi A, Yarmand MS, Razavi SH (2007) Effect of lecithin and calcium chloride solution on the microencapsulation process yield of calcium alginate beads. Iran Polym J 16:597–606
16. Allan-Wojtas P, Hansen LT, Paulson AT (2008) Microstructural studies of probiotic bacteria-loaded alginate microcapsules using standard electron microscopy techniques and anhydrous fixation. LWT-Food Sci Technol 41:101–108
17. Sheu TY, Marshall RT (1993) Microentrapment of lactobacilli in calcium alginate gels. J Food Sci 58:557–561
18. Park JK, Chang HN (2000) Microencapsulation of microbial cells. Biotechnol Adv 18:303–319