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CHARACTERISATION OF MICROENCAPSULATED *LACTOBACILLUS RHAMNOSUS* LR7 STRAIN*

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Survivability of free and microencapsulated *Lactobacillus rhamnosus* bacteria in different pH media was investigated. Microencapsulated bacteria were also subjected to freeze-drying. Encapsulation and freeze-drying are effective stabilisation methods increasing their resistance to environmental pH.

INTRODUCTION

Investigations are under way aiming at improved production methods of starter cultures, which constitute the most important element of probiotic preparations. The main objective of these studies was to extend the life span of bacterial cells in the product itself as well as in the gastrointestinal tract of humans and animals. This goal was achieved, among others, by stabilisation of probiotic bacteria on a carrier. Apart from the surface cell binding the most popular method of immobilisation includes placing the cells into the carrier matrix – in natural gels (such as agar, alginate, carrageen, collagen) and synthetic ones (polyacrylamide, polyurethane, polystyrene, polyurethane resins) or immobilising them in fibrous substances such as cellulose or cotton. Out of all methods of living cells' immobilisation, alginate and κ-carrageen gels are recommended for lactic acid bacteria immobilisation [Olejnik & Czaczyk, 1998]. The objective of this research project was to characterise *Lactobacillus rhamnosus* bacteria microencapsulated in alginate taking under consideration their survivability in pH found in different sections of the gastrointestinal tract. An attempt was also made to freeze-dry the microencapsulated bacteria.

MATERIAL AND METHODS

The strain of *Lactobacillus rhamnosus* LR7 bacteria used in these investigations was obtained from commercial starter of the company Rhodia Food BIOLACTA (Olsztyn, Poland) and the medium applied for their proliferation was MRS (Fluka). Bacteria from slants were rinsed with sterile distilled water and transferred onto 10 mL liquid substrate sterilised for 15 min at a temperature of 121°C and cultured for 48 h at 37°C. Inoculum prepared in this way was transferred in the amount of 4% into liquid MRS medium. After initial culturing for 48 h, the bacteria were encapsulated. Then, simultaneous incubation of free and encapsulated bacteria was carried out for 2 days. The polyanion used to form the outer surface of capsules was a sodium salt of alginic acid, type MV (SIGMA). The applied polycation forming the liquid core was a hydroxypropyloammonium starch dissolved in water (20°C) with 0.03 degree of substitution (Luboń Potato Company, Poland). The survival rate of the bacteria in microcapsules was measured after the initial volume of the bacteria culture was reached. Capsules (2 g) were dissolved using 200 mL of 0.5 N solution of sodium citrate. The survival rate of bacteria was tested in pH 2, 7, and 8. The pH of MRS Broth medium was brought to the value of 2, 7, and 8 using 1 N HCl and 0.5 N NaOH and stabilised by appropriate Titrisol buffers (Merck).

Through flooding method inoculation onto the plates using MRS Agar *Lactobacillus* bacteria survival rate was measured – both free and capsuled bacteria cells. All bacteria cultures were kept at a temperature of 37°C.

After 48 h the capsuled bacteria were freeze-dried in HETO MTC-5 type dryer for 36 h at a temperature of 23°C. After lyophilization, their survival rate was also measured using the flooding method inoculation onto Petrie's dishes. Humidity of the capsules was evaluated using the drying method. The capsules were dried at 60°C until they reached a constant mass. The above procedure was repeated twice.

RESULTS AND DISCUSSION

Parallel culture of free and encapsulated *Lactobacillus rhamnosus* bacteria

After the cultivation of the free and encapsulated bacteria was over the number of free and immobilised living bacteria was counted. This number was compared to the number of *Lactobacillus rhamnosus* bacteria after initial cultivation, which was 2.75×10^9 cfu/mL. This allowed concluding that the number of encapsulated bacteria grown

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under identical culturing conditions was higher in comparison with the number of free bacteria and reached 8.55×10^9 cfu/mL (Figure 1). The recorded differences between free and encapsulated bacteria cultures were statistically significant ($\alpha = 0.05$).

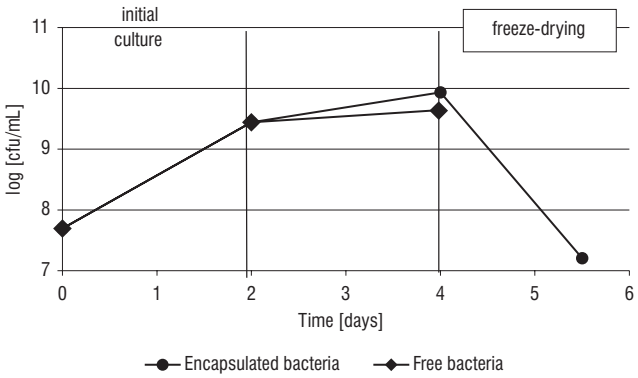

FIGURE 1. Growth of free and encapsulated *Lactobacillus rhamnosus* bacteria and effects of freeze-drying on survivability of encapsulated bacteria.

Figure 2 shows encapsulated bacteria directly after microencapsulation (a) and after 48 h of culturing (b).

Therefore, it was confirmed that it was possible to multiply *Lactobacillus rhamnosus* bacteria inside alginate capsules.

The above results were also corroborated by literature data [Champagne, 1994] that it is possible to manufacture lactic acid bacteria (including those potentially probiotic) inside capsules and balls formed from sodium alginate, while the presence of an immobilising factor exerts a positive impact on the multiplication rate of the bacterial biomass. However, on the basis of the studies of Champagne [1994], it is possible to assume that lactic acid bacteria are characterised by a considerable sensitivity to specific quantities of substrate and product and their high concentrations inhibit growth and biological activity of cells. The character of the gel used for bacteria immobilisation, especially the possibilities of controlling the substrate and product diffusion by the polymer membrane, creates conditions inside capsules or balls both for growth and development of metabolic processes of immobilised bacteria. An additional advantage is the fact that capsules protect bacteria against "violent" culturing environment associated with pH regulation, presence of oxygen as well as stirring the medium during the process of culturing.

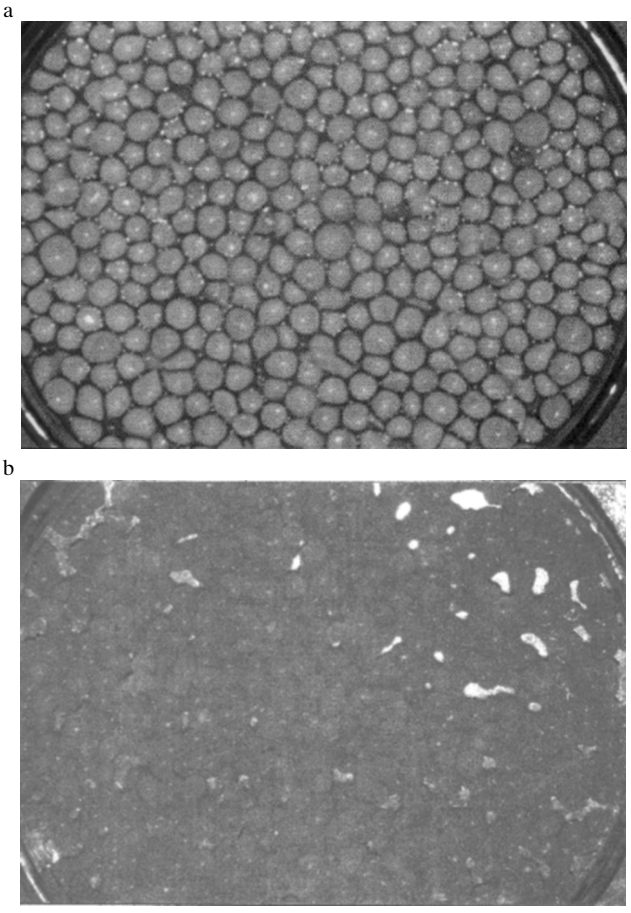

FIGURE 2. Capsules with bacteria directly after encapsulation (a) and after 48 h of culturing (b).

Shah and Ravula [2000] added to frozen fermented milk desserts (yogurt) *Lactobacillus acidophilus* MJLA1 and *Bifidobacterium* spp. BDBB2 probiotic bacteria which were encapsulated in calcium alginate and lyophilised. The survival rate of the bacteria added to the yogurt, which were cultivated for 12 weeks, was higher for the bacteria in microcapsules than in the case of the bacteria which were not microcapsulated. The encapsulation of *Bifidobacterium longum* B6 and *Bifidobacterium longum* ATCC 15708 in κ-carrageen also increased their survival rate in yogurt in comparison to the bacteria which were not microencapsulated [Adhikari *et al.*, 2000].

The results of the experiments presented in the paper correspond to the results found in the available literature [Champagne, 1994; Shah & Ravula, 2000; Adhikari *et al.*, 2000], although different bacteria types and cultivation conditions were used.

Incubation of free and encapsulated *Lactobacillus rhamnosus* bacteria in the environment with different pH

The comparative analysis of the survival rate of *Lactobacillus rhamnosus* bacteria numbers in free and encapsulated forms growing in media with pH 2, 7, and 8 at the temperature of 37°C for 48 h revealed that *Lactobacillus rhamnosus* bacteria survived longest at pH 7 and 8, *i.e.* pH characteristic for human saliva and that found in the duodenum. However, it should be emphasised that the number of encapsulated bacteria after 72 h of culturing at pH 7 and 8 reached 10^9 cfu/mL, *i.e.* it was higher by two orders of magnitude in comparison with free bacteria – the difference was statistically significant (Figures 4 and 5). The survival rate of *Lactobacillus rhamnosus* bacteria in pH 2 (Figure 3) was statistically significantly different from the survival rate of the bacteria at pH 7 and 8. After 48 h of culturing, the number of live free bacteria at pH 2 dropped to 0, while that of encapsulated ones remained at the level of 10^9 cfu/mL. After 72 h of culturing, all encapsulated bacteria were dead. Since food stays in the stomach not more than 3 h [Traczyk, 1992], the performed experiment demonstrated that both free and encapsulated bacteria were capable of surviving this period of time in the environment with pH 2, as numbers of live bacteria in the 3rd hour of culture were, respectively, 10^8 cfu/mL and 10^9 cfu/mL. In the environment with pH 7 or 8, *Lactobacillus rhamnosus*

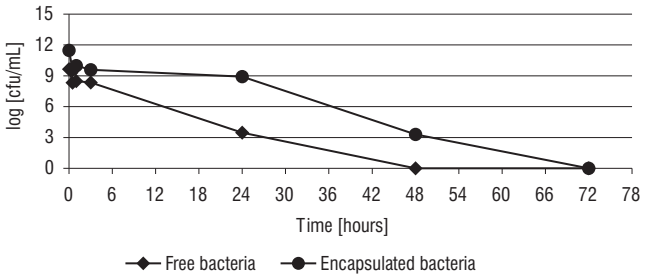

FIGURE 3. Survival rate of free and encapsulated *Lactobacillus rhamnosus* bacteria in the environment with pH 2.

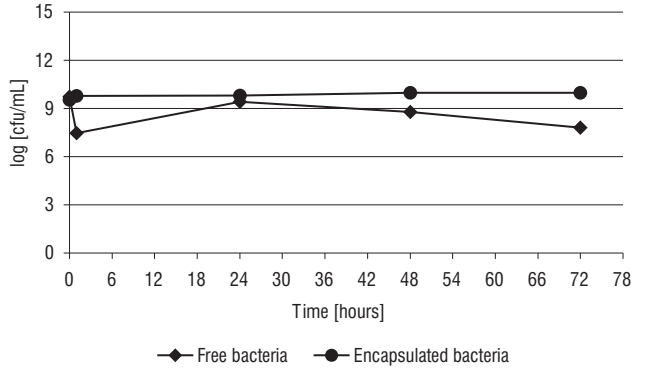

FIGURE 4. Survival rate of free and encapsulated *Lactobacillus rhamnosus* bacteria in the environment with pH 7.

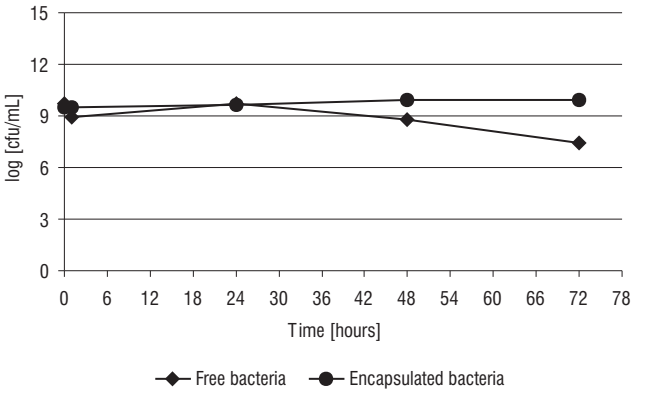

FIGURE 5. Survival rate of free and encapsulated *Lactobacillus rhamnosus* bacteria in the environment with pH 8.

bacteria remained alive longer than the time food stays in the mouth (10 min), or in the duodenum (1 h) [Traczyk, 1992].

Effect of freeze-drying on survivability of *Lactobacillus rhamnosus* bacteria

The analysis of encapsulated *Lactobacillus rhamnosus* bacteria survivability freeze-dried in a HETO MTC-5 drier for 36 h at 23°C showed that the number of bacteria, in the result of this process, decreased from 10^9 cfu/mL to 10^7 cfu/mL (Figure 1). The water content in the encapsulated bacteria after freeze-drying was 11%. When considering potentially therapeutic value of probiotic products and pharmacological products with intestine bacterial microflora one has to bear in mind that the product has to include a sufficient number of living and active cells when it is consumed (minimum 10^6 cfu/cm^3 of product) [Kołożyn-Krajewska, 2001]. The process of lyophilization reduced the number of living bacteria cells in the yogurt down to 10^7 cfu/mL, thus maintaining it at a level meeting the requirements with regard to a minimum number of cells in probiotic products.

Similar results were obtained for encapsulated *Lactobacillus* plantarum and *Bifidobacterium bifidum* dried with conventional and fluidal methods [Goderska *et al.*, 2001]. However, a comparative analysis of the above-mentioned three types of drying for *Lactobacillus rhamnosus* bacteria would be necessary.

CONCLUSIONS

1. *Lactobacillus rhamnosus* LR7 bacteria can be cultured inside alginate-starch capsules and such an immobilization system creates better conditions for bacterial growth in comparison with free bacteria culturing.
2. The encapsulation increases the resistance of the bacteria to different pH of medium environment.
3. The resistance of the bacteria to different pH of medium was increased by the encapsulation.
4. Freeze-drying is an effective stabilisation method of encapsulated bacteria.
5. Stabilisation of bacteria by microencapsulation gives a product easy to store and apply.

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CHARAKTERYSTYKA MIKROKAPSUŁKOWANEGO SZCZEPU *LACTOBACILLUS RHAMNOSUS* LR7

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W pracy postanowiono scharakteryzować mikrokapsułkowane bakterie *Lactobacillus rhamnosus* firmy Rhodia Food BIOLACTA (Olsztyn, Polska). Do utworzenia kapsułek zastosowano sól sodową kwasu alginianowego typu MV – zewnętrzna warstwa będąca polianionem i skrobię hydroksypropyloamoniową o stopniu podstawienia 0,03 – ciekły rdzeń będący polikationem. Kapsułki rozpuszczano przy użyciu roztworu cytrynianu sodu. Poprzez posiew na płytki metodą zalewową określano żywotność bakterii z rodzaju *Lactobacillus* zarówno wolnych, jak i kapsułkowanych. Postanowiono także ustalić wpływ kapsułkowania na przeżywalność bakterii w środowisku o pH 2, 7 i 8. Mikrokapsułkowane bakterie były także suszone sublimacyjnie.

Otrzymane wyniki badań udowodniły ochronny wpływ kapsułek na przeżywalność szczepu bakterii *Lactobacillus rhamnosus*. Zakapsułkowane bakterie *Lactobacillus rhamnosus* przeżywają w środowisku o pH 2 do 3 doby hodowli, a ilość żywych bakterii po 24 godzinie hodowli utrzymuje się na poziomie 10^8 jtk/mL. Bakterie wolne natomiast w 3 godzinie hodowli osiągają poziom 10^8 jtk/mL, a tym samym krótszy jest czas ich życia w środowisku o pH 2 (rys. 3). W środowisku o pH 7 bakterie wolne jak i kapsułkowane w 3 dobie hodowli osiągają poziom odpowiednio 10^7 jtk/mL i 10^9 jtk/mL. W środowisku o pH 8 w 3 dobie hodowli liczba żywych bakterii jest tego samego rzędu co w środowisku o pH 7 (rys. 4 i rys. 5). Suszenie sublimacyjne bakterii kapsułkowanych zmniejszyło ilość żywych komórek z poziomu 10^{10} jtk/mL na poziom 10^7 jtk/mL.

Mikrokapsułkowanie i liofilizacja tych bakterii okazały się efektywnymi metodami stabilizacji przez zwiększenie ich odporności na pH środowiska.