Preparation of Bacteria-Containing Microparticles using Water-Dispersed Polymers

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SUMMARY: Rhizobacteria-containing polymer microparticles have been prepared using either a complex coacervation (phase separation) method or a spray-drying technique. The microparticles obtained have been characterized as regards their particle size distribution, morphology and bacterial content. Long term bacterial survival within the microparticles stored under controlled conditions (relative humidity and temperature) has been investigated. Bacterial survival in the spray-dried microparticles has been found to be high over 6 months, when the residual humidity content within the particles is higher than ~ 20 wt %. Release of bacteria from the microparticles into an aqueous medium has also been studied. The release rate has been found to be fast, the total release being completed within 15 min. The release mechanism has been related to the coating structure and water affinity of the polymer particles.

Introduction

A great deal of interest has been paid recently to the applications of living microorganisms, such as bacteria, in the feed and seed industry. As for the seed industry, the inoculation of plants with rhizosphere-based bacteria for crop improvement and subsequent reduction of chemical pesticides has become a highly desired objective[1)]. In this case, bacteria act as a growth-stimulating agent and help fighting telluric pathogens. As far as feed is concerned, lactic bacteria can act as a probiotic additive which favours the growth of small animals such as piglets, just after weaning. However, in both cases, the expected beneficial effect of naked bacteria is greatly reduced due to their poor stability, either after inclusion into the seed coating or in the feed formulation[2)]. This behaviour is related to the death of bacteria mainly induced by the constraining humidity and temperature conditions during storage, processing and end-use. For this reason, the protection of bacteria through microencapsulation in polymer microparticles has recently been proposed as an original approach and studied in our laboratory, so as to increase the long term bacterial survival under various conditions.

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The aim of the present work is to prepare rhizobacteria-containing polymer microparticles, which will then be included in the seed coating or pelleting, so as to achieve direct bacterization of seeds (Fig. 1). Bacteria will then grow in or on the roots and will thus be able to have a growth-promoting effect on the plant. This strategy should make it possible to make a homogeneous deposit of the inoculum, at an optimal concentration, just nearby the roots of the seed, and to fulfill requirements about storage conditions, such as relative humidity, osmotic pressure, and oxygen levels, separately for seeds and bacteria.

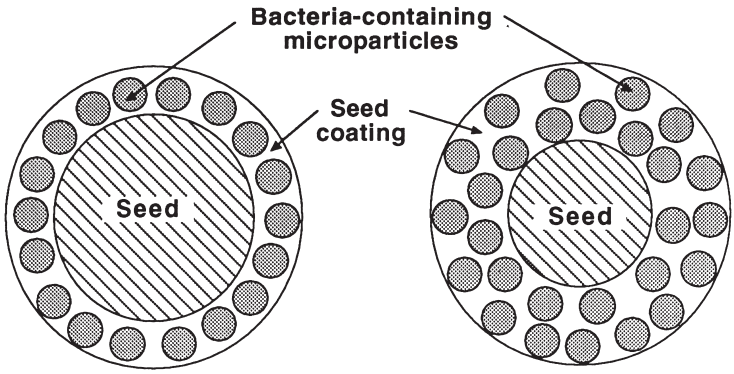

Fig. 1: Schematic representation for direct bacterization of seeds with bacteria-containing polymer microparticles included in the seed coating

Bacteria-containing microparticles were prepared either upon formation of polymer complexes from oppositely charged hydrocolloids, i.e. the so-called complex coacervation process, or upon spray-drying of polymer latex dispersions. The microparticles obtained were characterized as regards their particle size distribution, morphology and bacterial content. Long term bacterial survival within the microparticles stored under controlled conditions (relative humidity and temperature) was investigated. Release of bacteria from the microparticles into an aqueous buffered medium was finally studied, together with the water affinity of the polymer microparticles.

Experimental Methods

The rhizobacterium strain M3.1 used in this study belonged to the group *Pseudomonas fluorescens-putida*, Gram-negative and an aerobe rod, isolated from maize roots from Thailand. Bacteria grew for 7 days at 26 °C in a King's liquid medium B and were then

aseptically separated by centrifugation to a final concentration of ~ 5 x 10^{11} colony-forming units (cfu)/mL.

As for the complex coacervation process, bacteria were dispersed into either a paraffin oil suspension or the internal aqueous phase of a double water-in-oil-in-water (w/o/w) emulsion. The oily phases were then emulsified in water under stirring and coated with complex coacervates of gelatin (isoelectric point IEP = 7.8, bloom grade = 150) and polyphosphate (Hexatren® C5 SL, from Giulini Chemie) polyelectrolytes[3]. The coacervates were formed upon polymer phase separation induced by adjusting the pH to 4 and decreasing temperature to 5-8 °C. After adsorption on the surface of the oil droplets, the coacervates were crosslinked using glutaraldehyde to yield a continuous tough polymer membrane.

Bacteria-containing microparticles were also prepared using a spray-drying process of aqueous polymer dispersions[4]. These latexes contained acrylic and methacrylic ester copolymers (Eudragit® RS30D, from Röhm Pharma). The solid content of the dispersions was 30 wt%. Bacterial cells were added to the aqueous polymer dispersions under stirring before spray-drying. The inlet air temperature in the spray-drier was fixed between 60 and 80 °C and the spray feed rate ranged from 3 to 10 mL/min. In some trials, 4.5 wt% hydrophilic silica (Sipernat® 22, from Degussa) was added to the formulation to study the effect on long term survival.

Microparticle morphology was characterized using either optical microscopy (Nikon SMZ-U stereomicroscope) or Scanning Electron Microscopy (SEM) with low acceleration voltage (Jeol JSM 6301 F). Particle size distribution was determined using the Multisizer® Coulter counter and the Mastersizer® Malvern granulometer. Bacterial content was measured after dissolution or degradation of the polymer. Bacterial survival and residual humidity content were followed over long periods of time, up to 6 months, for microparticles stored under various conditions, so as to probe the effects of formulation, process parameters and storage conditions.

Results and Discussion

Starting from dispersions of bacteria in oil, complex coacervation of gelatin and polyphosphate led to bacteria-containing microparticles, whose mean diameter ranged ~ 230 µm. The bacterial content was found to be very low (~ 1.5 x 10^4 cfu/g of dried particles), compared to the initial bacterial concentration in oil (5 x 10^9 cfu/g of suspension). Moreover, the bacterial survival was poor after a few days. The strategy based on the w/o emulsion with bacteria incorporated in the dispersed aqueous phase gave even lower values of bacterial content and survival. These results were attributed to leakage of bacteria from the oil droplets during the preparation process and bacterial death upon storage. Leakage should arise from the hydrophilic character of the surface of the bacteria, which favours their migration to the outer

aqueous phase. Bacterial death is to be related to the low oxygen permeability of the polymer membrane[5], which is made of protein-rich complexes.

Good compatibility of bacterial strain with polymer dispersions was checked before spray-drying; concentration of bacteria in the Eudragit® polymer dispersions was found to range between 10^7 and 10^{11} cfu/g of polymer. Spray-drying of Eudragit® RS30D and bacteria dispersions made it possible to produce microparticles with a regular, smooth, spherical morphology, without any evidence of bacteria on their surface (Fig. 2).

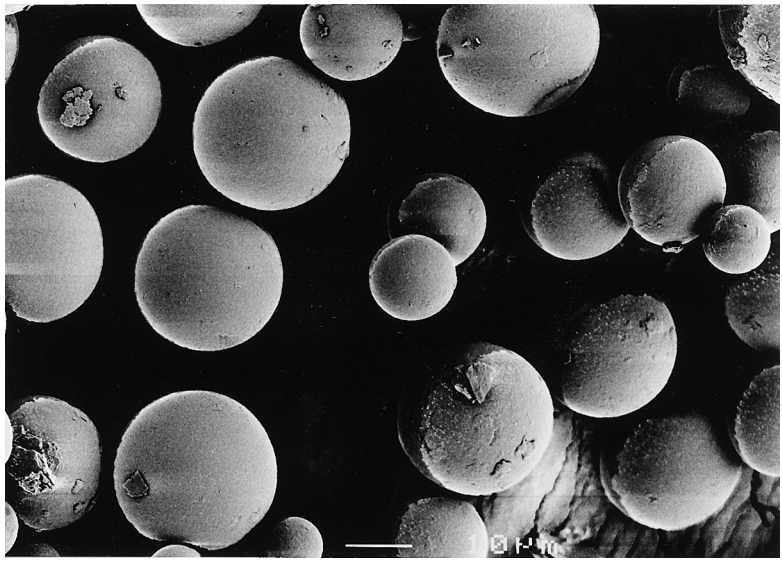

Fig. 2: SEM photomicrograph taken on gold-plated bacteria-containing polymer microparticles obtained by spray-drying of Eudragit® and bacteria dispersions

Using optical microscopy, it was shown that bacteria are only released after dispersion of the microparticles in ethanol. These observations led us to conclude to an effective encapsulation of bacteria within the polymer microparticles. The mean particle size was found to range ~ 35 µm, with a rather wide size distribution extending from 2 to 90 µm. Yield of microparticles was found to be higher than 85 % for a spray feed rate ranging from 3 to 10 mL/min and an inlet air temperature between 60 and 80 °C.

Fast release of encapsulated bacteria from Eudragit® microparticles was found to occur upon contact with an aqueous buffered medium. A burst effect was observed with an immediate release of ~ 10 % encapsulated bacteria. Release was shown to be complete in only 15

minutes. This behaviour was attributed to the high water affinity of the polymer coating. Due to the presence of hydrophilic cationic side groups at the surface of the initial latex particles, the polymer coating should likely contain preserved hydrophilic interfacial layers[6,7], which favours the diffusion of water within the bacteria-containing microparticles. The strong water affinity of the bacteria-containing microparticles was also confirmed by performing contact angle measurements of water droplets on pressed tablets of particles[8].

When the inlet air temperature of the spray-dryer was fixed at 60 °C, bacterial survival in the microparticles was found to remain high in ambient conditions. The content of living bacteria ranged ~ 10^6-10^9 cfu/g of Eudragit® polymer, in all the samples a few hours after preparation and even after a 2 month-storage period for silica-containing formulations. Long term survival was evaluated at 20 °C over 6 months under various controlled relative humidity (RH) conditions. It was found to be quite stable for RH values higher than 33 %, but to decrease rapidly for microparticles stored in a very dry atmosphere (0 % RH). Survival was shown to be clearly correlated to the residual humidity content in the powder. Finally, the addition of 4.5 wt % hydrophilic silica into the formulation was found to greatly enhance the long term survival of microencapsulated bacteria (Table 1).

Table 1. Long term survival of bacteria and residual humidity in Eudragit®-based microparticles stored under various RH conditions

Storage time (months)	Bacterial content / Residual humidity content log (cfu/g) / (wt%)				
	0 % RH	33 % RH		55% RH	100 % RH
	no silica	no silica	silica	no silica	no silica
0[a]	7.3 / 27	5.8 / 33	8.3 / 27	5.8 / 34	8.5 / 34
1	5.5 / 11	1.0 / 14	8.2 / 25	4.6 / 19	9.0 / 33
3	4.5 / 12	- / 10	7.0 / 21	3.6 / 23	8.3 / 37
6	2.4 / 13	- / 10	6.8 / 14	- / 12	8.0 / 45

[a] Between 4 and 8 hours after preparation

Conclusion

Feasibility of polymer microparticles containing living bacteria was investigated using either coacervation of polyelectrolyte complexes or spray-drying of polymer dispersions. Complex coacervation did not lead to a satisfactory encapsulation yield and bacterial survival. On the contrary, feasibility was demonstrated using the spray-drying technique. Microparticles obtained were thoroughly characterized and shown to fulfil the main requirements to be included in a seed coating or pelleting. Their size is lower than 100 µm, the production yield and bacterial content are high, and the bacterial survival is preserved over long periods of time at a level which is consistent with the targeted application. The predominant effect of residual humidity within the microparticles on the long term survival of bacteria was clearly evidenced. Finally, the contact with an aqueous medium was shown to trigger the release of bacteria from the microparticles, and the release rate was found to be very fast. This feature has now to be adjusted by modifying the polymer, so as to limit the leakage of bacteria from the microparticles during the seed coating or pelleting. Moreover, the spray-drying process and the formulation have to be worked out, so as to eliminate the caking of the powder occurring in the spray-dryer.

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