THE STORAGE EFFICIENCY OF IMMOBILIZED *Bradyrhizobium japonicum* STRAIN USING ENCAPSULATION METHOD

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SUMMARY

There is a growing need for new formulations of carriers with better protection for bacterial inoculum. One of the newer techniques in inoculum making is encapsulation method. With this method, the whole bacterial cells are immobilized in defined space – matrix, where the cells are protected from environmental activities before use. Encapsulation of the inoculum was performed with ionic gelation method. The alginate-based microparticles (500-600 µm) containing viable B. japonicum strain were solidified in $CaCl_2$. The initial number of viable bacteria in every sample was 9.0 log CFU/ml. Chitosan coated particles had a higher mortality rate than non-coated particles, with 1.3 log CFU/ml in lyophilized and wet microparticles stored at room temperature. High viability of B. japonicum was registered in wet particles stored at constant -20°C for thirty days with a viability rate of 8.84 log CFU/ml.

Key words: *Encapsulation, Bradyrhizobium japonicum, storage, viability, biofertilizers*

INTRODUCTION

One of the basic methods in agricultural production is seed or soil inoculation by microorganisms. This method has been known since 19th century when Beijernick isolated and described the microorganisms that form nodules on legume roots (Crawford et al., 2000). The goal of the inoculation technique is to provide adequate numbers of live and effective rhizobial bacteria for colonization of soil and plant root, which will result in the rapid start of root nodulation. Better nodulation will have result in increased N_2 fixation, which leads to increased growth of crop yield (Catroux et al., 2001; Deaker et al., 2004). Moreover, the majority of commercial bacterial inocula are peat-based (Denton et al., 2009). The peat as a carrier for microorganisms can be easily applied as a coating on legume seeds or be used for direct application in the soil. The peat-based formulations showed good results, especially with rhizobial inoculum (Thompson, 2013; Denton et al., 2009). The disadvantage of conventional peat-based inocula is its longevity and protection from environmental factors during storage before use and during application in the soil (Fallik and Okon, 1996; Bashan, 1998).

In the last decades, there has been an increased tendency in searching for new formulations of the carriers. The aim of bioencapsulation is protection of living bacteria before and during introduction into the soil, as well as their slowed and prolonged release in the seedbed (Bashan,1986; Kim et al.,2012; Schoebitz et al., 2013). The advantages of encapsulation are better protection from biotic and abiotic stresses, shelf life stability and reduced possibility of contamination during storage time and transportation, as well as controlled release of microorganisms due to soil degradation (Bashan et al., 2002). However, it is very costly compared to peat-based carriers and it requires more

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biotechnological expertise. The difference between conventional and encapsulation methods is that in the microparticles the whole bacterial cells are immobilized in defined space and the cells are protected from external effects of the environment (Karel et al.,1985;Rathore et al., 2013). On the other hand, in the conventional method, the viable bacterial cultures are directly put and mixed into the commercially available carriers and they do not provide sufficient protection against external conditions. With encapsulation method, the cells are immobilized within the particle which can be made from biodegradable polymer materials. The particle itself has a permeable structure that allows access of nutrients, exchange of the gases and metabolites for maintaining the viability of immobilized cells (Ding and Shah, 2009; John et al., 2011; Rathore et al., 2013). Alginate is the most common polymer for construction of macro- or microcapsules in many industries and it is extracted from brown algae. The alginate is a linear macromolecule consisting of two monomers linked via β - (1-4)-D-Mannuronic acid to α - (1-4) - L- Guluoronic acid (Schoebitz et al., 2013). The main purpose of alginate based microparticles is to protect bacterial cells from the environment and to maintain their viability during the storage time (Young et al., 2006). There are various formulations and methods for microparticles preparation. The most recognized and economically advantageous method in the agricultural and food industry is ionic gelation. For the pre-encapsulation process, the sodium alginate solution is thoroughly mixed with cell culture. The produced droplets are then mixed with cation donor solution, i.e. $CaCl_2$ where particles obtain their spherical shape (Cassidy et al., 1996). The particle formation happens almost instantly when the contact between sodium alginate and Ca^{2+} ion occurs. Na-alginate solidification of the particles is possible because Ca^{2+} ions react with negatively charged alginate chains and for this reason, the three-dimensional structure is made. This structure contains mainly water as part of the gel through which Ca^{2+} is diffused into the droplet and networks it via crosslinking "from outside to inside" (Vemmer and Patel, 2013). One more protection against mechanical stress during storage is adding an outer layer or extra coating on the particles (Zaeim et al., 2017). With ionic polymer coating, the morphology of the particle is defined as suspension of polyanionic part – the core of the particle (sphere) and polycationic part – the shell of the particle (capsule). In this case, the polyanionic solution is chitosan (0.2 % solution). Between chitosan's amine groups and alginates carboxyl groups complex formation is made, which adds more protection to the living cells inside the particles (Poncelet et al., 2001). The aim of this study was to make small-sized (500-600 µm) calcium alginate microparticles (spheres and capsules) which contain viable cells of commercial *Bradyrhizobium japonicum* 344 strain. By supplementing microparticles with an extra chitosan layer, it is considered that the microparticles would get extra protection during storage time. Since one of the most restrictive effects in bacterial inoculation production is high cell morality of bacteria during the storage time, the principal aim of the study was to determine the encapsulated cell viability at different storage temperature conditions, and to determine which one is the most adequate.

MATERIALS AND METHODS

Medium and bacterial strain
The medium used for bacterial growth was YMB (Yeast Mannitol Broth). The composition per liter was: 0.5 g K_2HPO_4; 0.1 g NaCl; 0.2 g $MgSO_4$; 10 g mannitol; 5 ml bromothymol blue; 0.4 g yeast extract. Rhizobial strain used for encapsulation was *Bradyhrizobium japonicum* 344 which was obtained from strain collection of University of Zagreb, Faculty of Agriculture, Department of Microbiology.

Preparation of strain sample for encapsulation
In 40 ml of sterile YMB solution, pure culture of *B. japonicum* 344 strain with a sterile loop was added. The bacterial sample was grown in incubation shaker (Orbital Shaker-Incubator ES-20, Biosan) for 7 days (150-200 rpm, 28 °C).

The encapsulation method and microparticles formation
The initial step of encapsulation process was adding 40 ml of bacterial suspension (10^9 CFU/ml) grown in YMB into 500 ml Erlenmeyer flask with 160 ml sodium alginate. Sodium alginate was prepared ahead by totally dissolving it in distilled water. The suspension of bacteria and Na-alginate was thoroughly mixed. Encapsulator B-390 (Büchi, Switzerland; provided from Department of Chemistry, Faculty of Agriculture, University of Zagreb) was used for the formation of micro-droplets. The formation of droplets was conducted in these conditions: gas (nitrogen) pressure was set to 27–28 mbar, at a frequency of 800 Hz in a sterile environment. The diameter of the nozzle was 300 µm. Under the nozzle, 500 ml glass flask was placed, which contained 0.1 mol dm^{-3} of $CaCl_2$ solution (100 ml $CaCl_2$). When droplets of Na-alginate bacterial suspension dripped into the $CaCl_2$ solution, they immediately formed as microspheres. For full formation of the microparticles, the glass flask was placed on a magnetic stirrer (Tehtnica Železniki MM-540) and stirred at low speed for 40 minutes. The encapsulation method yielded about 50 grams of

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Na-alginate microparticles. The beads were transferred from $CaCl_2$ to sterile gauze and carefully rinsed with saline solution (0.85 % (w/v) NaCl). One-half of the spheres were coated additionally with 100 ml of 0.2 % polysaccharide chitosan solution to obtain microcapsules. The microspheres and microcapsules were placed at various temperatures in Petri dishes.

Microparticles storage at different temperatures
Wet sodium alginate microparticles were stored at -20 °C and 7 °C, likewise they were stored at room temperature as wet and as lyophilized microparticles. The growth rate of rhizobial bacteria was determined from zero until the thirtieth day (days of observation were 0, 1, 2, 4, 6, 8, 10, 12, 14, 20, 22, 30). One part of calcium alginate particles was stored at -20 °C and bacterial viability was observed only on zero and thirtieth day. Wet chitosan microparticles and lyophilized ones were stored at the same conditions described above.

Cell count and morphology of microparticles
The cell viability was determined for thirty days after the process of encapsulation except for one sample that was stored at -20°C, where enumeration of the cells was conducted only on the first and last day. The capsules with entrapped bacteria had to be dissolved in a tube that contained 8 ml of saline solution (0.85 % (w/v) NaCl) with added 1 ml Na-tricitrate for an easier count of viable bacteria. The enumeration was carried out only with living cells by serial dilution method. Liquid bacterial inocula (0.1 ml of bacterial solution) was plated on YMA (Yeast Mannitol Agar) and incubated for 5-7 days at 30 °C. After incubation the growth rate of *B. japonicum* 344 was determined and the results were expressed as CFU (Colony Forming Units)/ml. The results are presented as log 10 of an average of three repetitions. Morphology of microparticles was observed under the optical and fluorescence microscope (OlympusBX60).

RESULTS AND DISSCUSION

Number determination of *B. japonicum* 344 and microparticles morphology
Initial culture concentration of *B. japonicum* 344 after encapsulation in calcium alginate microspheres as well as calcium alginate microcapsules was 9.0 log CFU/ml. Samples from different storage temperatures were taken from zero to 30 days and the microparticles morphology and bacteria viability were determined. It was noted that Ca-alginate microspheres had a well-defined membrane (Fig. 1a-d). Encapsulation process decreases the viability of a certain amount of bacteria (Bashan et al., 2002; Ivanova et al., 2005). With the method of encapsulation, the spherical microparticles with a diameter of 500-600μm were obtained. This particle size has a higher bacterial survival rate, because of good mechanical strength, better diffusion, as well as nutrient and metabolite modification with optimal oxygen intake (Uludag et al., 2000; Rathore et al., 2013). Chitosan was added as an additional layer protector around the microsphere membrane to improve the storage capacity of the particle itself and it directly affected the survival of the bacteria. Under the microscope, a slight deformation of the membrane of the microcapsule was shown, already after the first day (Fig. 1e). It is evident that with the increased storage time, the chitosan-coated microparticles membranes became more deformed, affecting the survival of the bacteria inside them (Fig. 1f).
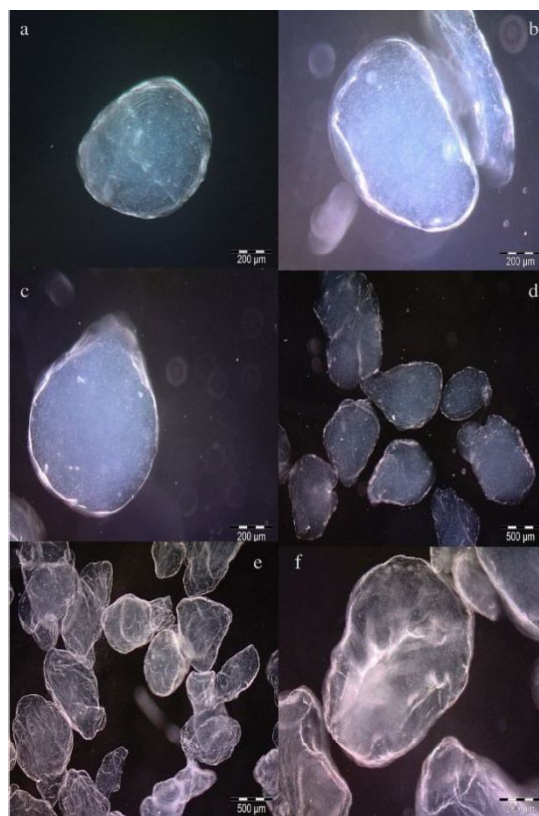
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Figure1. Microparticles stored at + 7 ° C, (a) day zero, (b) tenth day, (c) eighteen day, (d) twentieth day (a-d: Na-alginate); (e) first day, (f) twentieth day (e-f: chitosan coated)

Viability of *B. japonicum* 344 in Ca-alginate microspheres at different temperatures
It is shown in Fig. 2 (Appendix 1 - Tab. S1) that after the initial number of bacteria (9.0 log CFU/ml), every sample had the same number of bacteria on the first day, except wet Ca-alginate microspheres stored at -20 °C, with numbers dropping by 1 log. The decreasing trend was observed in every given sample but the most significant decline in Ca-alginate microspheres was observed in a wet stored sample at -20 °C. After ten days, in the same sample the number of bacteria decreased to 5.67 log CFU/ml. On day 20, viability was reduced to almost 63 % of their initial number. On the last day of sampling (day 30) the viability of bacteria was 2.33 log CFU/ml. Wet and lyophilized microspheres stored at room temperature had similar results in the reduction of bacterial viability. On the second day of sampling, microspheres contained 8.67 log CFU/ml of bacteria. In wet Ca-alginate particles, a slightly greater decline in bacterial numbers was observed. More noticeable decrease in numbers was observed in lyophilized microspheres between 14th and 20th day. The number of bacteria reduced from 6.33 log to 5.33 log CFU/ml. On the last day of observation of the above-mentioned samples, the final number of bacteria was 4.33 log CFU/ml for both samples. The lowest decline observed in Ca-alginate microspheres which were subject to constant environmental changes during storage was in a wet sample stored at +7 °C. The decline of bacterial numbers was noticed on 4th day (8.67 log CFU/ml). A large decrease was observed between the days 6 and 8 where the viability decreased from 8.67 log to 7.7 log CFU/ml. The constant CFU was observed between the days 20 and 22 (6.33 log CFU/ml). On the last day, there was a slight decrease in bacterial number, with result of 6.00 log CFU/ml.

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Ca-alginate microspheres

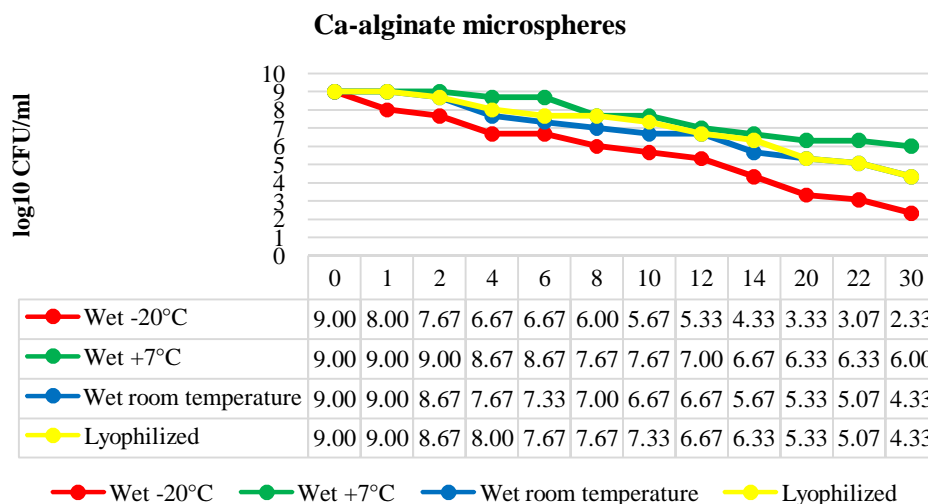|	0	1	2	4	6	8	10	12	14	20	22	30
Wet -20°C	9.00	8.00	7.67	6.67	6.67	6.00	5.67	5.33	4.33	3.33	3.07	2.33
Wet +7°C	9.00	9.00	9.00	8.67	8.67	7.67	7.67	7.00	6.67	6.33	6.33	6.00
Wet room temperature	9.00	9.00	8.67	7.67	7.33	7.00	6.67	6.67	5.67	5.33	5.07	4.33
Lyophilized	9.00	9.00	8.67	8.00	7.67	7.67	7.33	6.67	6.33	5.33	5.07	4.33

Figure 2. *B. japonicum* 344 viability during 30 days of storage in Ca-alginate microspheres at different temperatures and under the different storage conditions (log CFU/mL)

The highest number of viable bacteria was observed in wet Ca-alginate microparticles stored at -20 °C from zero to 30th day (data not shown in graphs). The decline in viability was less than 1 log whereas the initial 9.0 log decreased to 8.48 log CFU/ml. The particles were stored at a constant temperature, and the bacteria had slow vital functions. Since the other microparticles were under greater influence of temperature change, this had a significant effect on the decrease of their viability, as well as on the structure and permeability of the particles themselves, resulting in reduction of the protection for bacteria from external influences.

Viability of *B. japonicum* 344 in Ca-alginate microspheres coated with chitosan at different temperatures
Ca-alginate microspheres coated with chitosan showed a greater decline in bacterial viability than in Ca-alginate microspheres. As it is demonstrated in Fig. 3 (Appendix 1 - Tab. S2), on the first day of sampling the bacterial numbers decreased (8.33 log CFU/ml in every sample). The decrease in bacterial number in microspheres was within similar ranges. It was observed that the wet capsules stored at -20 °C had slightly higher bacterial number than the rest of the samples. The final number at -20°C storage samples was 3.33 log CFU/ml, which is by 1 log greater than the same sample but without chitosan coating. The second highest viability was observed in wet chitosan coated microspheres stored at +7 °C. The decline between zero days of sampling (9.0 log CFU/ml) and the last day of sampling (2.33 log CFU/ml) was significant. The number of viable bacteria was reduced by 74.11 %. An even greater decline in bacterial numbers was noted in wet and lyophilized microcapsules stored at room temperature. Their number of alive bacteria decreased from 9.0 log CFU/ml to 1.33 log CFU/ml, which is a decline of 85.22 %. There are several reasons why chitosan coated microparticles affected the number of bacteria. One of the reasons for reduced bacterial viability in particles coated with chitosan is deformation of the membrane capsule itself. The second reason for capsule deformation is that chitosan does not enter deep into the alginate structure but is accumulated on the outside (Gaserod et al., 1998). Another reason is the competence between the Ca^{2+} ions and the chitosan amino group. The Ca^{2+} immediately hardened, and complex coacervation formation was made between chitosan and alginate. A porous gel structure was formed, and chitosan molecules came into contact with bacteria after a certain time (Gaserod et al., 1998; Wittaya-Arekul et al., 2006). Probably the main reason for decreasing of bacterial number is that chitosan has an antimicrobial effect as it affects the cell membranes and damages the membrane function (Raafat et al., 2008).

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Ca-alginate microspheres coated with chitozan

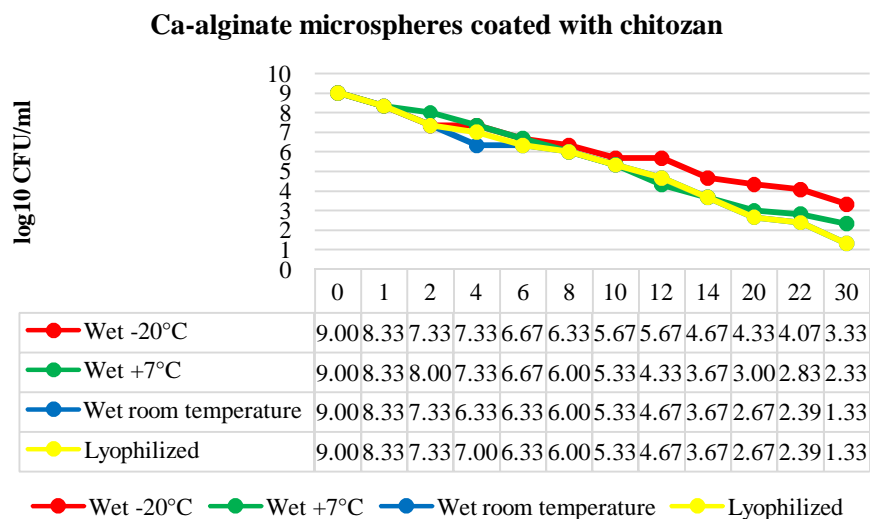|	0	1	2	4	6	8	10	12	14	20	22	30
Wet -20°C	9.00	8.33	7.33	7.33	6.67	6.33	5.67	5.67	4.67	4.33	4.07	3.33
Wet +7°C	9.00	8.33	8.00	7.33	6.67	6.00	5.33	4.33	3.67	3.00	2.83	2.33
Wet room temperature	9.00	8.33	7.33	6.33	6.33	6.00	5.33	4.67	3.67	2.67	2.39	1.33
Lyophilized	9.00	8.33	7.33	7.00	6.33	6.00	5.33	4.67	3.67	2.67	2.39	1.33

Figure 3. *B. japonicum* 344 viability during 30 days of storage in Ca-alginate microspheres coated with chitosan at different temperatures and different storage conditions (log CFU/ml)

CONCLUSION

In conclusion, microparticles stored at + 7°C significantly differed from other samples. In order to yield a sufficient number of viable bacteria immobilized within calcium alginate microparticles, the storage temperature should be low and constant. Also, the consistent environment factors during the storage time are also important in the production of every available commercial inoculant. Coating of Ca-alginate microparticles with chitosan did not prove to add protection to the bacteria – on the contrary, the number of bacteria was lower than in Ca-alginate microspheres. This was a preliminary study, and the fact that should be taken into account is that encapsulation methods in agriculture are still developing and are not at the industrial level. Additional investigations should be carried out, involving other species and strains of soil-borne bacteria with further laboratory experiments and field trials, which should be conducted under natural conditions.

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APPENDIX 1

Table S1. *B. japonicum* 344 viability during 30 days of storage in Ca-alginate microspheres at different temperatures and under different storage conditions (log CFU/mL).

Storage time (Days)	–20 °C	+7 °C	Room temp.	Lyophilized
0	1.00E+09	1.00E+09	1.00E+09	1.00E+09
1	1.00E+08	1.00E+09	1.00E+09	1.00E+09
2	7.00E+07	1.00E+09	7.00E+08	7.00E+08
4	**7.00E+06[a]**	**7.00E+08[a]**	7.00E+07	1.00E+08
6	**7.00E+06[a]**	**7.00E+08[a]**	4.00E+07	7.00E+07
8	3.70E+06	7.00E+07	1.00E+07	7.00E+07
10	7.00E+05	7.00E+07	7.00E+06	4.00E+07
12	6.70E+05	3.70E+07	7.00E+06	3.40E+07
14	4.00E+04	7.00E+06	7.00E+05	4.00E+06
20	**4.00E+03[a]**	**4.00E+06[a]**	4.00E+05	4.00E+05
30	**4.00E+02[a]**	**1.00E+06[a-c]**	**4.00E+04[b]**	**4.00E+04[c]**

Values superscripted with the same letter within the row are significantly different according to the posthoc Tukey HSD test (p<0.001).

Table S2. *B. japonicum* 344 viability during 30 days of storage in Ca-alginate microspheres coated with chitosan at different temperatures and different storage conditions (log CFU/ml).

Storage time (Days)	–20 °C	+7 °C	Room temp.	Lyophilized
0	1.00E+09	1.00E+09	1.00E+09	1.00E+09
1	4.00E+08	4.00E+08	4.00E+08	4.00E+08
2	4.00E+07	3.70E+08	4.00E+07	4.00E+07
4	4.00E+07	4.00E+07	4.00E+06	1.00E+07
6	7.00E+06	**7.00E+06***	4.00E+06	4.00E+06
8	4.00E+06	1.00E+06	1.00E+06	3.70E+06
10	7.00E+05	**4.00E+05***	4.00E+05	4.00E+05
12	7.00E+05	**4.00E+04***	**7.00E+04***	**7.00E+04***
14	7.00E+04	**7.00E+03***	**7.00E+03***	**7.00E+03***
20	4.00E+04	**3.70E+03***	**7.00E+02***	**7.00E+02***
30	4.00E+03	**4.00E+02***	**4.00E+01***	**4.00E+01***

Values superscripted with * are significantly different, compared to the analog storage conditions of microspheres at the relative time, according to the posthoc Tukey HSD test (p<0.001). No significant differences were found between the different storage conditions of microcapsules.

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