Impact of carrier material on fermentative activity of encapsulated yoghurt culture in whey based substrate

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Abstract

The main objectives of this paper were to study the influence of the carrier material used for encapsulation and of bead size to fermentative activity and viability of the dairy starter culture Lactoferm ABY 6. Encapsulation of yoghurt culture in beads with diameter of 1 mm provides better results than encapsulation in beads with larger diameter. Alginate beads and chitosan coated beads have proved to be a strong barrier for nutrients from substrate, so samples with those beads have lower viable cell count, lower titratable acidity and higher pH value after 5h of fermentation at 42 °C, than samples with WPC-alginate beads. Also those beads have significantly ($P < 0.05$) lower cell leaking, than WPC-alginate beads and lower antioxidant capacity. Encapsulation of yoghurt culture in WPC-alginate carrier with diameter of approximately 1mm provided the best characteristics for fermented product. Samples with these beads have significantly ($P < 0.05$) higher increase of viable cell number after fermentation, despite of major cell leaking (19.7%). Moreover, sample with these beads have the highest titratable acidity, the lowest pH value after fermentation (the best fermentative activity) and the best antioxidant characteristics.

Keywords: whey, encapsulation, alginate, chitosan, WPC, fermentation.

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Whey generated in the process of cheese production is a major by-product of the dairy industry.

Whey represents a very good substrate for use in a various biotechnological processes. In recent years, the bioconversion of whey has become an interesting process that achieves reduction of environmental pollution with the production of microbial biomass and metabolites. Fermentation of whey by probiotic yoghurt cultures could be an alternative for increasing the amount of exploited whey and incorporation of this by-product in human nutrition.

Modern consumers are increasingly interested in their personal health and expect their food to be healthy and to prevent illness [1]. There are numerous known bacterial species, which beneficially affect the balance of more than 400 different microorganisms that naturally inhabit the human digestive system. Various types of probiotic bacteria include *Lactobacillus* and *Bifidobacterium* as the most used species. [2] The potential health benefits of probiotics are: anti-infection properties, anticarcinogenic and antimutagenic properties, serum cholesterol reduction, immune system stimulation, alleviation of lactose intolerance and nutritional enhancement [3,4]. Many reports indicated that there is poor survival of probiotic bacteria in products containing free probiotic cells [5]. To have their beneficial effects on the host's health probiotic bacteria must stay alive until they reach their site of action.

Encapsulation of bioactive components can be used in many applications: masking flavours or colours, controlling oxidative reaction, extending shelf life, providing sustained and controlled release. Probiotic encapsulation is primarily used to protect the cells against adverse environment [6] and only secondarily to control release. Most probiotics are very sensitive to environmental conditions, so development of enhancing probiotic viability techniques is highly necessary [7,8]. In dairy industry, immobilization has been applied to improve survival and delivery of bacterial cultures [9]. Many studies have examined the different carriers in order to find the most suitable material for a particular culture and substrate. There are many studies for encapsulation different culture for cow's milk fermentation [10], usually used alginate [11] as carrier.

Alginate is the most widely used encapsulating material for encapsulation of cells and bioactive compounds. Encapsulation of probiotic bacteria in alginate is possible due to it is non-toxic and rapid method. Nevertheless, the use of alginate is limited due to its low stability and fast degradation in the presence of chelating agents. Some polycations, such as chitosan,

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form strong complexes with alginate. Chitosan is biodegradable and biocompatible. It also has antibacterial activity due to which it is more frequently used as external shell in capsules made with polymers such as alginate, than as carrier [9].

Whey proteins are less used encapsulating material. They have high nutritional value and ability to form gels, emulsions, and foams. The ability of whey proteins to form gels and microcapsules without the use of severe heat treatment and any chemicals makes them an attractive material for controlled delivery applications in food industry [12]. Whey Protein Concentrate (WPC) is used as an effective carrier for the protection of sensitive functional and bioactive components such as probiotic organisms in a range of functional food [13].

This work is a continuation of the study about the application of whey as functional fermented beverage. The aim of study is to determine the influence of types of carrier material and bead size on the fermentation activity and cell viability in order to obtain a functional beverage.

EXPERIMENTAL

Culture and media

The mixture of *Streptococcus salivarius ssp. thermophilus* (80%), *Lactobacillus acidophilus* (13%), *Bifidobacterium bifidum* (6%), *Lactobacillus delbrueckii ssp. bulgaricus* (1%) is used for this work. It is commercial lyophilized dairy starter culture (Lactoferm ABY 6, Biochem s.r.l. Monterotondo, Roma, Italy). The culture was stored according to the manufacturer's instructtions at –18 °C until use (no longer than 20 months).

Whey and sterile cow's skim milk were obtained from domestic dairy plant Imlek a.d. (Belgrade, Serbia). The chemical composition of whey was: total solids 9.8±0.03%; protein 2.6±0.012%; fat 1.05±0.08% and lactose 5.6±0.114 %. The chemical composition of milk was: fat 0.5%; proteins 2.9%; carbohydrates 4.7% and calcium 0.12%. After collection, the whey was stored at –18 °C until use.

Encapsulation

Alginate beads with mean diameter 0.76±0.09 mm were produced using an electrostatic extrusion technique [14]. Sodium alginate (Sigma–Aldrich) was dissolved in distilled water and pasteurized at 70 °C for 30 min. Dairy starter culture (1%) was diluted in 100 mL of whey and mixed with 150 mL of the sodium alginate solution. Alginate–cell suspension containing 1.60% sodium alginate was added dropwise by syringe pump (Racel, Scientific Instruments, Stamford, CT, USA) through a needle tip to the solution of 2% $CaCl_2$ (Acros organics, USA). Alginate drops solidified upon contact with $CaCl_2$ and formed beads with entrapped bacterial cells. The beads were allowed to harden in gelling solution for 30 min, and after that washed with sterile physiological solution (0.85% NaCl) to remove excess calcium ions and free cells. The alginate beads with mean diameters greater than 2 mm were produced without application of electrostatic potential during encapsulation process. The same technique was used for production of WPC-alginate beads with the following modification: 2% ABY-6 culture diluted in 50 mL of whey, mixed with 50 ml 15% WPC solution and then mixed with 150 mL of sodium alginate solution. Whey protein concentrate (WPC 80, 80% protein based on dry weight) manufactured from sweet whey (DMV International, The Netherlands) was used. Alginate beads contain 1.60% alginate; WPC-alginate beads contain 1.60% alginate and 2.4% whey proteins. The beads were stored at 4±1 °C in yeast extract solution (0.2%) until use.

Coating drops

Coating procedures were performed according to Zhou et al. [15] methods. 0.4 g of low-molecular-weight chitosan (Acros organics, USA) was dissolved in 90 mL distilled water acidified with 0.4 mL of glacial acetic acid to achieve a final concentration of 4 g/L. The pH was then adjusted to between 5.7 and 6.0 by adding 1 M NaOH. The mixture was filtered through Whatman #4 filter paper. Volume was adjusted to 100 mL before autoclaving at 121 °C for 20 min. 15 g of washed beads were immersed in 100 mL of chitosan solution with gentle shaking at 100 rpm for 40 min on an orbital shaker for coating. The chitosan-coated beads were washed and used instantly.

Fermentation

Medium used for fermentation contained 70% whey and 30% sterilized milk. Whey was pasteurized for 60 min at 60 °C in water bath before fermentation. Based on preliminary experiments (data not shown) 30% milk was used for beverage formulation as concentration that appropriate for sensory quality improvement. Sample was inoculated by adding 6% ABY-6 commercial culture encapsulated in alginate, chitosan-alginate, and WPC-alginate beads.

After inoculation samples were incubated at 42 °C. Fermentations were stopped by quick cooling after 5 h when pH between 5.0 and 4.5 was reached for most samples.

Viable cell enumeration

Cell number was determined by pour plate counting method. M17 agar has been used as substrate for *S. salivarius ssp. thermophiles* and MRS agar has been used as substrate for probiotic bacteria: *Lb. delbrueckii ssp. bulgaricus, Lb. acidophilus* and *Bifidobacterium*

bifidum [16]. The results are presented as the total number of viable cells on both substrate (M17 and MRS) and cell number expressed in log CFU/mL for free cells and log CFU/g for encapsulated cells.

Determination of cell leaking

The cell leaking was determined by the formula:

$$\text{Cell release}(\%) = \frac{\text{Free cell number}(\text{CFU})}{\text{Free cell number}(\text{CFU}) + \text{Cell number in beads}(\text{CFU})} \times 100 \quad (1)$$

Titratable acidity and pH

Titratable acidity was determined by the Soxhlet––Henkel method and obtained in Soxhlet–Henkel degrees (°SH) [17]. The pH value was measured using a pH meter (Inolab, WTW 82362, Wellheim, Germany) at room temperature.

DPPH free radical scavenging activity assay

Radical scavenging activity was determined using a 1.1-Diphenyl-2-Picrylhydrazyl (DPPH) free radical scavenging assay according to the method described by Bulatović *et al.* [18].

The beads size analysis

The diameters of randomly selected beads of each treatment were measured with optical microscope (Carl Zeiss Microscopy GmbH, UK) using magnification of 2.5× and 5×.

Statistical analysis

Experiments were performed in triplicate. All values are expressed as mean ± standard deviation. Mean values were analysed using one-way ANOVA. The Tukey post hoc test was performed for means comparison (Origin Pro 8 computer package, Origin Lab Co., Northampton, USA). Data was considered significantly different when $P < 0.05$.

RESULTS AND DISSCUSSION

The shape and dimensions of the particles are shown in Fig. 1. The beads were globular in shape. The diameter of uncoated alginate beads was 0.76±0.09 mm and 2.42±0.07 mm, which was significantly lower than diameter of coated alginate beads (1.18±0.08 mm and 3.01±0.11 mm). Diameter of WPC-alginate beads was slightly bigger to the diameter of alginate beads: 0.94±0.07 mm and 2.55±0.09 mm.

Viability and leaking

Encapsulation of the bacterial culture brings many benefits. Fermentation of whey by commercial cultures designed for yoghurt production could be an interesting way for including whey in human consumption. Encapsulated cells are protected from external factors, so they have a greater ability to survive adverse environmental conditions. Also cells can be isolated from the original substrate and used again for fermentation or incorporated into another product (chocolate, ice cream, etc.). For bacterial encapsulation it is necessary to make an appropriate balance between the protection of the bacteria from the negative impacts from outside and the passage of nutrients necessary for the life of bacteria.

Because of difficulties in the exchange of nutrients between medium and encapsulated cells inside of beads, fermentations with encapsulated cells require more time than with free cells. This work investigated which carrier and which particle size is the most suitable for the fermentation of whey based substrate. The cell numbers before and after fermentation for all samples are shown in Table 1.

Data from Table 1 show that small beads had significantly ($P < 0.05$) higher increase in the number of viable cells then large beads for all samples. That can be explained by nutrients exchange. Viable cells in small particles are closer to the surface which is in contact with the substrate, than in large beads. The cells placed inside the large particles have very limited access to nutrients in substrates. WPC-alginate beads had the highest increase in viable cell number ($P < 0.05$) within all examined particles. That can be explained by the positive impact of WPC on growth of yoghurt culture. This is important because probiotic living cells in yoghurt enhance its therapeutic value. [19,20].

Numerous studies and epidemiological data suggest the importance of antioxidants when in prevention of many diseases. Food antioxidants prevent oxidative processes only in fatty substances in the course of food production and food storage; natural antioxidants are classified as “bioactive substances” and play an important role in cell metabolism [21].

There are a large number of scientific reports that provide evidence of the health benefits of microorganisms including their production of antioxidants [22]. As shown in Table 1, during the fermentation process, the percentage of inhibition of DPPH radicals significantly ($P < 0.05$) increased from an initial value of 15.1% to variety of values recorded after the fermentation, just as expected on the basis of previous research [23,18]. The percentage of inhibition of DPPH radicals was approximately 34.9±0.5% for all samples except samples with WPC-alginate beads. Samples with WPC--alginate beads had slightly higher inhibition of DPPH radicals, for both size of beads. The obtained results are in accordance to the results reported by other

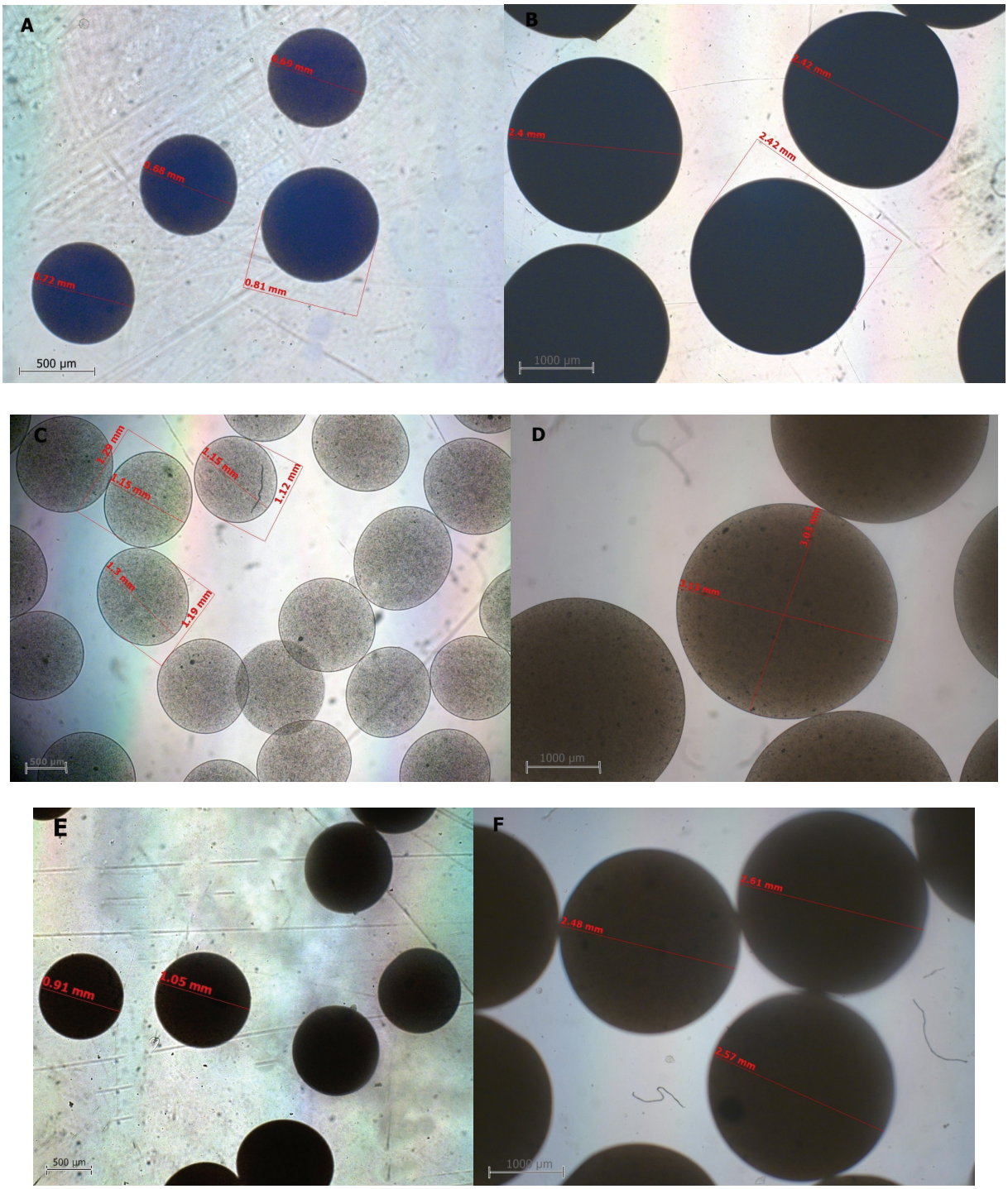

Figure 1. Small alginate beads (a), large alginate beads (b), small alginate beads coated with chitosan (c), large alginate beads coated with chitosan (d), small WPC-alginate beads (e), large WPC-alginate beads (f); optical microscope, magnification: 5× for small beads and 2.5× for large beads).

researchers [24] who found that the metabolic products of LAB obtained by utilization of oligosaccharides contribute to the higher antioxidant activity of yoghurt prepared by *S. thermophilus, L. delbrueckii ssp. bulgaricus* and *L. plantarum.* Also, it could be assumed that proteolysis [25] and lactic acid production [26] as the results of microbial activity during fermentation could be additional sources of antioxidant activity.

Cell leaking is shown in Table 2. Number of leaked cells in the fermentation medium was 3.44±0.14% of the total bacterial population for sample with small alginate beads and 5.32±0.15% for large alginate beads.

Table 1. Effect of carrier material and beads size on the viable cell count (log CFU/g) nd DPPH scavenging activity during 5 h fermentation at 42 ℃; ⊿log represents increase in viable cell number (⊿log= viable cell number after fermentation, log CFU/g – viable cell number before fermentation, log CFU/g); DPPH before fermentation was 15.1±1.1% for all samples

State or parameter	Alginate small beads	Alginate large beads	Alginate-chitosan small beads	Alginate-chitosan large beads	WPC-alginate small beads	WPC-alginate large beads
Before fermentation	7.8439	8.0402	7.6981	7.59106	7.4487	7.6652
After fermentation	8.9685	8.34635	9.0050	8.47129	8.9699	8.9614
⊿log	1.1246	0.30615	1.3069	0.88023	1.5212	1.2962
DPPH inhibition, %	35.4±1.0	34.5±1.3	34.8±0.9	34.4±1.2	36.5±1.1	38.8±1.4

Table 2. Effect of carrier material and beads size on cell leaking in samples during 5 h fermentation at 42 ℃

Leaking	Alginate beads	Alginate –chitosan beads	WPC-alginate beads
From small beads, %	3.44	2.48	19.7
From large beads, %	5.30	5.02	23.5

Slightly lower cell leakage (2.48±0.14% of the total bacterial population) was observed for sample with small alginate-chitosan beads, and 5.02±0.18% for large alginate-chitosan beads. Number of leaking cells in samples with WPC-alginate beads was significantly (P < 0.05) higher than in other samples: 19.7±0.21 for small beads and 23.5±0.23 for large beads. Based on the results, it could be said that chitosan and alginate develop a complex that reduces the porosity of alginate beads and decreases the leakage of the encapsulated probiotic. On the other hand, based on great cell leaking can be said that WPC building complex with alginate makes the particles more porous. These results are consistent with other research that shows that coating the beads with chitosan gives better protection to probiotic cells than alginate non coated beads [27–30].

Titratable acidity and pH value

Table 3 shows two important fermentation parameters: pH and titratable acidy. Beverage is considered to have a good quality if it has a titratable acidity of approximately 44 °SH [17]. Since whey is a poor substrate, yoghurt culture produces significantly less lactic acid, even if it is not encapsulated.

Titratable acidity of beverage with small alginate beads was 14.0 ˚SH and slightly lower 12.0 °SH for large alginate beads. Chitosan coated beads had slightly lower titratable acidity: for small beads it was 13.0 °SH and for large beads 11.6 °SH. Small WPC-alginate beads had the best titratable acidity of 15.4 °SH, which is significantly (P < 0.05) higher then titratable acidity of sample with large WPC-alginate beads 11.2 °SH. It was also significantly (P < 0.05) higher then every other examined sample (alginate beads both size, alginate-chitosan beads both size). This indicates that samples with small WPC-alginate beads could have the best taste and the best protection (because lactic acid has protective effect for product) in relation to the other five types of beads. At the end of fermentation, pH was 4.90 for sample with small alginate beads and 4.96 for sample with large alginate beads. The sample with chitosan coated beads has significantly higher pH value 5.15 for small beads and 5.30 for large beads. Sample with small WPC-alginate beads had the lowest pH value of 4.85. Small WPC-alginate beads have shown the best fermentative ability: the sample with these beads has the highest titratable acidity, and the lowest pH value and it also has the highest increase in viable cell number. This is important because the sensory quality is a major factor for consumer acceptance [31]. Other studies have also demonstrated that encapsulating probiotic with whey protein can increase viable cell number [31–35]. Despite the high percentage of leakage, the greatest number of viable cells remains in these particles. Good fermentative parameters can be explained by the highest increase in encapsulated viable

Table 3. Effect of carrier material and beads size on titratable acidity and pH value during 5h of fermentation at 42°C

Parameter	Alginate small beads	Alginate large beads	Alginate-chitosan small beads	Alginate-chitosan large beads	WPC-alginate small beads	WPC-alginate large beads
Titratable acidity before fermentation, °SH	4.2	4.2	4.2	4.2	4.2	4.2
Titratable acidity after fermentation, °SH	14.0	12.0	13.0	11.6	15.4	11.2
pH before fermentation	6.29	6.29	6.29	6.29	6.29	6.29
pH after fermentation	4.90	4.99	5.15	5.30	4.85	5.30

cell number and also the highest number of free cells in substrate. These free cells can also perform fermentation without barriers in the nutrients exchange. In production of fermented probiotic beverages, there is no need for removing viable cells from the medium, so cell leaking is not an unwanted factor. In this regard, it can be said that WPC-alginate beads have shown the best characteristics for production of fermented beverage of all beads considered in this work.

CONCLUSION

The results have shown that different carrier material provides positive effect for different use of encapsulated cells. The results show that the bead diameter is in correlation with leaking and fermentative ability. All beads with diameter between 0.76-1.13 mm have shown better fermentative activity and less cell leaking than beads with the same carrier material and diameter between 2.44–3.03 mm. The study has indicated that coating alginate beads with chitosan, reduces the porosity of beads and decreases the leakage of the encapsulated probiotic during the fermentation in comparison with not coated alginate beads. This type of particles is suitable for operation in systems where particles are subjected to a large number of washes, or systems that require as little as possible of free viable cells in the medium.

WPC-alginate beads have shown the best fermentative activity. Despite of high percentage of leakage, those beads have the highest number of viable cells after fermentation. These particles are suitable for the production of fermented dairy products because they allow the best growth of yoghurt culture and parameters of the product (pH and titratable acidity). Additionally, samples with these beads show good antioxidant characteristics.

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IZVOD

UTICAJ NOSAČA NA SPOSOBNOST FERMENTACIJE IMOBILISANE JOGURTNE KULTURE U SUPSTRATU NA BAZI SURUTKE

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(Naučni rad)

Osnovni cilj ovoga rada jeste određivanje uticaja vrste nosača i veličine čestica na tok fermentacije supstrata na bazi surutke pomoću komercijalne jogurtne kulture. Jogurtna kultura je mešavina četri vrste bakterija mlečne kiseline od čega su tri probiotske: *Streptococcus salivarius ssp. thermophilus* (80%), *Lactobacillus acidophilus* (13%), *Bifidobacterium bifidum* (6%), *Lactobacillus delbrueckii ssp. bulgaricus* (1%). Rad je nastavak istaživanja moguće primene surutke u proizvodnji funkcionalnog fermentisanog proizvoda. Odnos surutke i mleka 70:30 je najoptimalniji za postizanje najboljih senzornih svojstava. U radu su ispitivane tri vrste nosača: alginat, algint obložen hitozanom i mešavina alginata i proteina surutke u odnosu 2:3, kao i dve veličine čestica: prečnika približno 1 mm i između 2,4 i 3,0 mm. Manje čestice svih ispitivanih nosača pokazale su bolje osobine u svim ispitivanim oblastima: pH vrednost uzorka nakon fermentacije bila je niža, titracijska kiselost viša, porast broja živih ćelija tokom fermentacije viši i otpuštanje ćelija iz čestica bilo je niže. Alginat obložen hitozanom pokazao se kao najteža barijera za nutritijente iz supstrata ali i najbolja zaštita za ćelije. Otpuštanje ćelija iz ovih čestica je bilo najniže (2,48% kod malih i 5,02% kod velikih čestica), ali je i porast broja živih ćelija nakon fermentacije bio najniži od svih ispitivanih uzoraka. Alginatne čestice su takođe pokazale nizak procenat otpuštanja ćelija (3,44% kod malih i 5,30% kod velikih čestica). Najbolje karakteristike za korišćenje u proizvodnji fermentisanog napitka pokazale su WPC-alginatne čestice prečnika 0,94±0,07. Ove čestice su i pored velike poroznosti (procenat otpuštenih ćelija je iznosio 19,7%) pokazale najviši porast broja živih ćelija tokom fermentacije (Δlog 1,512), što je i najbitniji faktor kada su u pitanju probiotski proizvodi. Takođe su pokazale i najveću promenu parametara pokazatelja toka fermentacija: promena titracijske kiselosti i pH vrednosti uzoraka sa ovim česticama bila je najizraženija.

Ključne reči: Surutka • Inkapsulacija • Alginat • Hitozan • WPC • Fermentacija