Original Article

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Effect of starch and glycerol on the properties of alginate-microcapsules creating by phase separation coacervation method

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ABSTRACT

The aim of our research was to prepare probiotic microcapsules with alginate, starch, glycerol and to evaluate effect of different microcapsules formula on their properties. *Lactobacillus acidophilus* ATCC 4356 were encapsulated in alginate microcapsules by phase separation coacervation method. The mixture of 3% sodium alginate, 5% or 10% starch, 15% glycerol and cell biomass were dripped into 2% calcium chloride solution (all concentration were in % w/v) and the newly formed microcapsules were incubated for approximately 30 mins in order to obtain the rigid structure. After freeze-drying of the microcapsules, the more spherical shape and higher bacteria density were observed with the formula of alginate-starch-glycerol in compare to the formula without glycerol. The images from the scanning electron microscope (SEM) of cross section of those microcapsules with glycerol showed a uniform concentric circle inner layers, which was not seen in the formula without glycerol. Both starch and glycerol improved the survival of *L. acidophilus* after freeze-drying process and help to maintain low moisture of obtained microcapsules. After 3 months storage at 4°C, the viability of *L. acidophilus* in both types of microcapsules decreased but remained at 10^8 (cfu/g) colonies forming unit per gram. The highest bacteria density of 1.91×10^9 cfu/g were observed with the formula of alginate-glycerol-10% starch as well as the virtually constant low water activity (0.011).

1. INTRODUCTION

Probiotics products are very popular nowadays because they contain microorganisms which are beneficial to the digestive system. To maintain the quality of the products, one of the most important and challenging criteria is the viability of the microorganisms when exposed to acidic environment in the gastrointestinal tract. Microencapsulation followed by freeze-drying of the product is the most common method which currently used to protect bacteria from external effects. In order to enhance bacteria viability during the microencapsulation as well as freeze-drying process, protective agents such as starch and glycerol are commonly added in the formula [1-3].

The aim of our study was to prepare encapsulated *Lactobacillus acidophilus* microcapsules by the phase separation coacervation method together with freeze-drying the obtained products. Moreover, the effect of additional starch and glycerol in the formula were evaluated using several criteria such as external shape, internal structure, bacteria density, moisture content and water activity immediately after freezing and during storage. The results showed that the obtained microcapsules could be use as the raw materials in pharmaceutical production.

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2. MATERIALS AND METHODS

2.1. Materials

L. acidophilus ATCC 4356 were kindly provided by Microbiology Department of National Institute of Drug Quality Control. Sodium alginate (Alg), tapioca starch (Starch), glycerol (Gly), De Man-Rogosa-Sharpe (MRS) broth and agar were purchased from Titan (India) and all were autoclaved at 116^{o}C for 20 minutes.

2.2. Methods

2.2.1. Biomass collection:

L. acidophilus ATCC 4356 was cultured in 200 ml MRS broth at 37^{o}C in 5% CO_2 incubator (Sanyo; Japan). The cell density which measured by optical density at 270 nm with spectrophotometer (Hitachi; Japan) must reached 0.95 – 1.05, which equivalent to 10^{10} - 10^{11} cfu/g or wet biomass greater than or equal to 3.0 g. Then, cell biomass was collected by centrifugation at 4,000 rpm for 10 minutes.

2.2.2. Microencapsulation method:

Encapsulated *L. acidophilus* microcapsules were prepared by phase separation coacervation method with addition of glycerol and starch with various concentration to the formula. 3.0 g of cell biomass together with 5 or 10% starch, 15% glycerol were resuspended in 100 ml of 3% sodium alginate. The suspension was pumped through a 25-gauge needle at 60 - 80 drops per minute into a 2% calcium chloride solution to obtain the microcapsules. After 30 minutes incubation at room temperature, the microcapsules were freeze-dried by Alpha Christ 1-2LD plus freeze dryer with following parameters: pre-cooling at -80^{o}C for 4 hours and freeze-dried at -40^{o}C, 0.001 mbar for 24 hours.

2.2.3. Determination of bacteria density:

Approximately 1.0 g of microcapsules were dissolved in 0.1 M sodium citrate to release the cell from encapsulated alginate matrix. The solution was then undergone a serial dilution and 0.5 ml of solution at 10^{7} – 10^{10} times dilution was plated on MRS agar, incubated at 37^{o}C in 5% CO_2 incubator overnight. The overnight grown colonies were counted and calculated into cfu/g and log cfu/g unit.

2.2.4. Moisture content and water activity of the microcapsules:

The moisture content of obtained microcapsules was determined by heating 0.5 – 1.0 g of sample at 105^{o}C for 3-5 minutes and then calculated the water mass loss due to drying.

The samples were sent to Center for Biotechnology (COB), Viet Nam Institute of Dietary supplement (VIDS) for determination of water activity on Hygrolab water activity meter.

2.2.5. Microscopic structure of L. acidophilus microcapsules:

The stereo microscope (Nikon; Japan) was used to obtain the outer shape as well as cross section image of the microcapsules at the magnification of 25X. The inner structure of the microcapsules was observed using a cross section under the scanning electron microscope (Nikon) at magnification from 3,000X to 7,500X.

2.2.6. Stability of L. acidophilus microcapsules during storage:

L. acidophilus microcapsules were stored in sealed aluminum bags at 2 – 8^{o}C, 25% humidity. The moisture content and bacteria density were determined using the method in 2.2.3 and 2.2.4 at certain storage time for up to 90 days.

3. RESULTS

3.1. Effect of glycerol on the structure and bacteria density of *L. acidophilus* microcapsules

According to previous research, 15% glycerol keep the bacteria survive during freeze-drying process[1] while 5 – 10% starch added to the formula maintain round shape of microcapsules after freeze-drying[2]. The effect of glycerol to the microcapsules structure and shape were studied using the method in 2.2.1 without the cell biomass. The results were described in Table 1 and Figure 1 for 10% starch without or with 15% glycerol formula, which abbreviated as Alg-Starch10 and Alg-Gly-Starch10 respectively.

When applying both formula with cell biomass added, the bacteria density and moisture content of obtained microcapsules when were showed in Table 2. While the moisture content of both samples was virtually the same, the number of bacteria in Alg-Gly-Starch10 microcapsules were 10.35×10^9 cfu/g, 10 times higher than in Alg-Starch10 formula (7.65×10^8 cfu/g)

Table 1. Criteria for evaluation of adherence to antibiotic prophylaxis guidelines

	Alg-Starch10	**Alg-Gly-Starch10**
Shape and surface	Almost rounded, rough surface, distort shape (Figure 1 a1 and 1 a2)	Mostly rounded and sphere, smooth surface (Figure 1 b1 and 1 b2)
Physical condition	Crunchy and easy to break Hard to make a cross section	Soft but more elastic Easy to make a cross section
Cross section	Different but cluttered layers (Figure 1 a3)	Virtually uniform concentric layers (Figure 1 b3)
Moisture content after freeze-drying (%)	1.01 ± 0.08	1.08 ± 0.11

Table 2. Bacteria density and moisture content of Alg-Starch10 and Alg-Gly-Starch10 microcapsules after freeze-drying

	Bacteria density (cfu/g)	**Moisture content (%)**
Alg-Starch10	$7.65 \pm 1.94 \times10^8$	1.01 ± 0.08
Alg-Gly-Starch10	$10.35 \pm 3.15 \times10^9$	1.08 ± 0.11

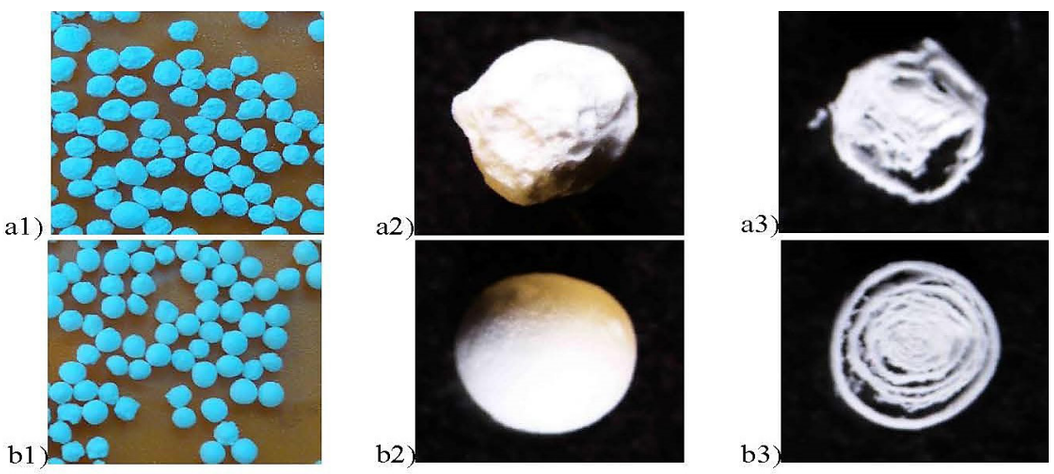

Figure 1. Images of freeze-drying microcapsule under stereo microscope with 25X magnification. (a) Alg-Starch10 microcapsules. (b) Alg-Gly-Starch10 microcapsules.

3.2. Effect of starch concentration on the structure and bacteria density of *L. acidophilus* microcapsules

The images of *L. acidophilus* microcapsules with 5% or 10% starch concentration in the formula (Alg-Gly-Starch5 and Alg-Gly-Starch10, respectively) under the SEM showed the structure with different layers, which consisted a thick outer shell and many concentric inner layers (Figure 2 a1, 2 a2, 2 b1 and 2 b2). The whole structure of the microcapsule was further made of diagonal network of alginate matrix which strengthened by inter-bridged linkage between starch particles. In 10% starch microcapsules, the inner layers were more rounded, the whole structure was denser compared to the lower concentration samples. At the magnification of 3,000X – 7,500X, *L. acidophilus* cell which attached to the surface of starch granules (Figure 2 a3 and 2 b3) could be visibly seen.

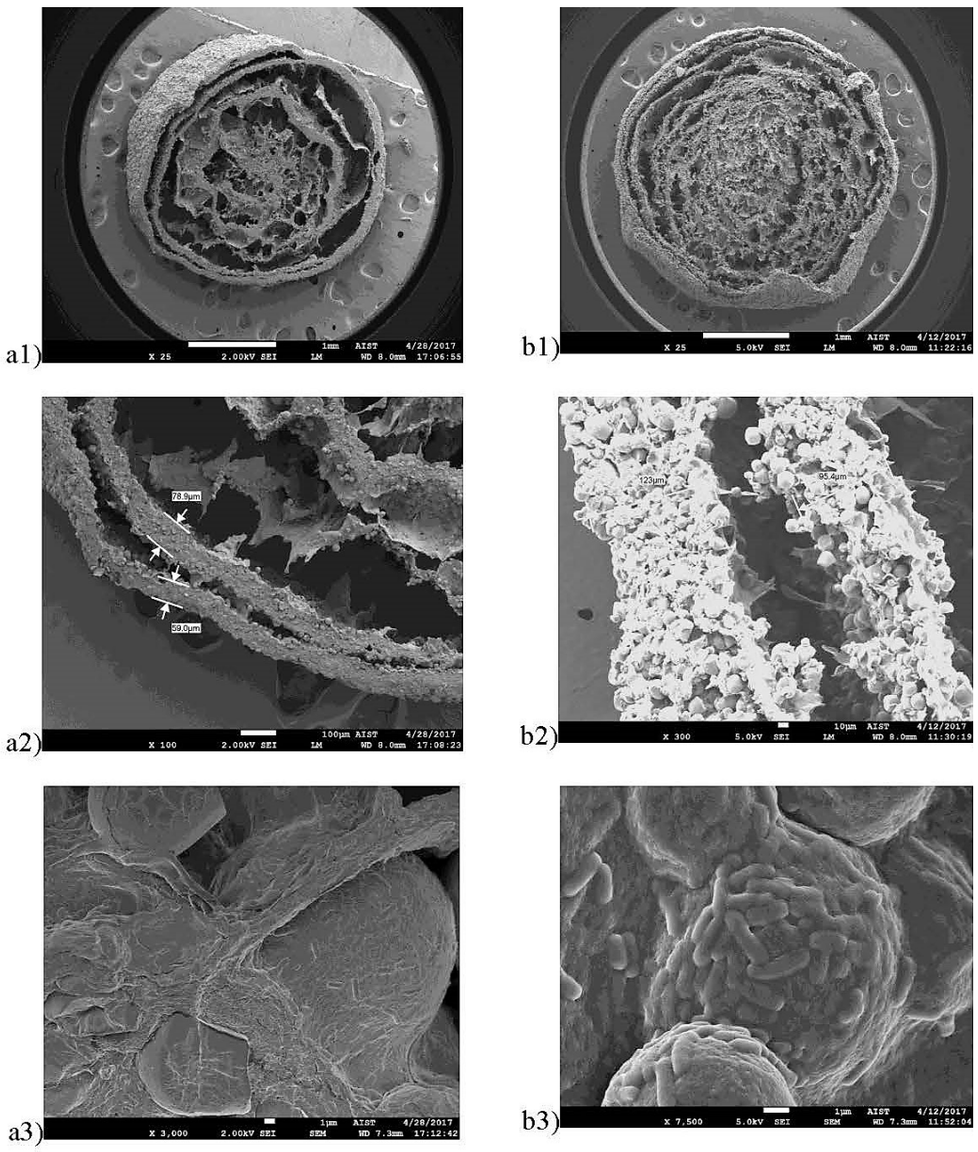

Figure 2. ISEM images of Alg-Gly-Starch5 (a) and Alg-Gly-Starch10 (b) microcapsules
a1, b1: Cross section at the mafnification of 25X
a2, b2: Outer shell of the microcapsules at the magnification of 100X - 300X
a3, b3: *L. acidophilus* attached on the surface of starch granules at the magnification of 3000X - 7500X

Before freeze-drying, the number of bacteria in both Alg-Gly-Starch 5 and Alg-Gly-Starch10 samples were approximately 3.18×10^{10} cfu/g (10.5 in log cfu/g) and 22.29×10^{10} cfu/g (11.3 in log cfu/g), respectively (Figure 3). After freeze-drying, the bacteria density in the Alg-Gly-Starch5 microcapsule were decreased approximately 10-fold to 3.51×10^{9} cfu/g (9.5 in log cfu/g) while the number of bacteria in Alg-Gly-Starch10 microcapsules remained at 1.04×10^{10} cfu/g (10.0 in log cfu/g).

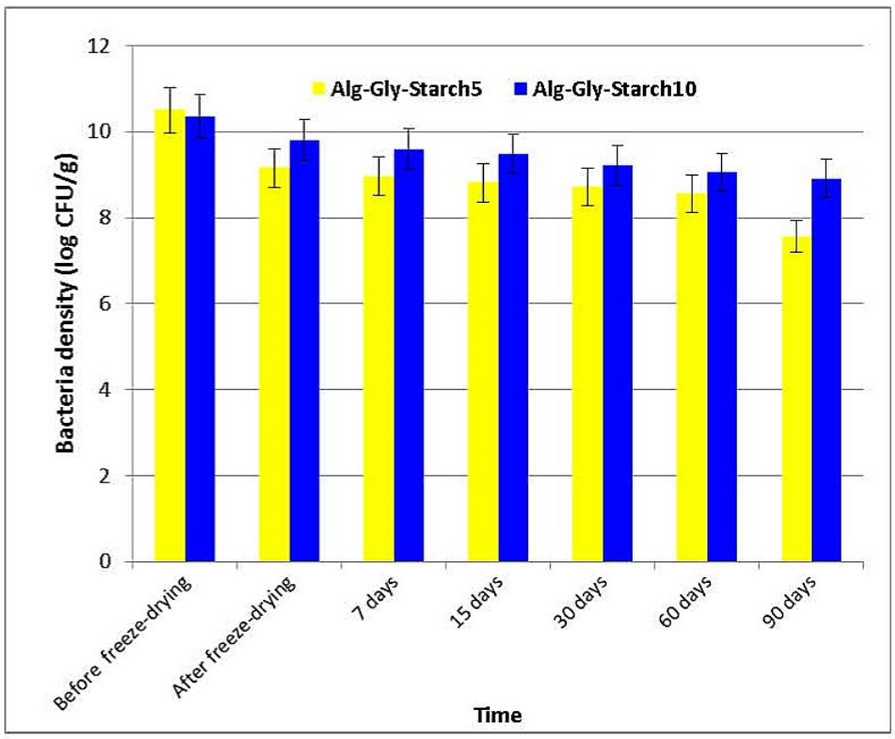

Figure 3. Bacteria density in Alg-Gly-Starch5 and Alg-Gly-Starch10 microcapsules before, after and during storage

3.3. **Effect of starch concentration on bacteria density of *L. acidophilus* microcapsules during storage time**

After 3 months at storage condition, the bacteria density in both Alg-Gly-Starch5 and Alg-Gly-Starch10 microcapsules were dropped to 1.62×10^{8} cfu/g (8.2 in log cfu/g) and 1.91×10^{9} cfu/g (9.3 in log cfu/g), respectively.

The same trend could be seen with the water activity (a_w) of both samples. Immediately after freeze-drying, a_w of Alg-Gly-Starch5 samples were approximately 0.102, which were much higher than 0.032 of Alg-Gly-Starch10 samples. After 90 days of storage, the a_w of the two samples decreased approximately 2-fold to 0.056 and 0.011 (Table 3).

Table 3. Water activity (a_w) of microcapsules samples during storage

	Storage time	Water activity (a_w)
(days)	Alg-Gly-Starch5	Alg-Gly-Starch10
0	0.102	0.032
60	0.054	0.011
90	0.056	0.011

4. DISCUSSIONS

4.1. Effect of glycerol on the structure and bacteria density of *L. acidophilus* microcapsules

In phase separation coacervation method, the addition of starch helped to enhance the gel network structure[1]. Moreover, the addition of 15% glycerol helped the microcapsules surface less shrinkage and smoother while the inner structure formed the more uniform concentric layers, which were consistent with previous study by Dejardin et al.[4]. According to Morgan et al.[5], during the pre-cooling phase, high viscosity of the suspension which used for phase separation process ensured that all the particles in the formula were fixed in the gaps between the glass phase. When the water sublimation process occurred, the structure could be well maintained. In Sapana et al. research[6], glycerol in the formula increased viscosity of the suspension and also affected the transition temperature when ice particles sublimated during freeze-drying, reducing the shrinkage of microcapsules surface.

The similar moisture content of all microcapsule samples showed that glycerol had little effects on the water sublimation process during freeze-drying. However, bacteria density was much more drastically affected. In Alg-Gly-Starch10 microcapsules with 10% starch in the formula, the number of bacteria were 10 times higher than in non-glycerol samples. The same results were observed with 2% starch in the formula from Phuong et al. research[1]. Morgan et al.[5] and Weng et al.[7] argued that glycerol acted as a membrane permeabilizer, which redistributed water within the cell and reduced the amount of bound water on the protein of the cell surface. Therefore, it helped to reduce cell membrane damage which lead to better protection of the bacteria during freeze-drying.

4.2. Effect of starch concentration on structure and bacteria density of *L. acidophilus* microcapsules

In microcapsules formula with starch concentration of 5% and 10%, the outer appearance of all samples was virtually rounded and less distort. The SEM images showed that Alg-Gly-Starch10 microcapsules displayed denser structure than the Alg-Gly-Starch5 microcapsules due to the ability to fill in the hollow pores of the alginate matrix by starch particles. The results were consistent to Xing et al.[8] study with starch concentrations up to 14%.

At magnification of 3,000X and 7,500X, the SEM images visibly showed *L. acidophilus* cell adhered to the surface of starch granules. Crittenden et al.[3] also explained that starch particles provided the surface for probiotics to adhere and the higher starch concentration, the higher number of *L. acidophilus* cell could be bound to them (in 10% samples the bacteria density were 10 times higher than in 5% samples).

4.3. Effect of starch concentration on bacteria density of L. acidophilus microcapsules during storage time

In the formula with starch at all concentration, the bacteria were better protected during storage time. After 3 months of storage at 4°C, the bacteria density in all samples decreased but remained sufficiently at 10^8 cfu/g. Although microcapsules with higher starch concentration (Alg-Gly-Starch10) provided higher bacteria density and lower water activity after freeze-drying and during storage, further evaluation were necessary to obtain an optimize concentration.

5. CONCLUSION

Using phase separation coacervation method, our study successfully prepared the encapsulated alginate microcapsules which contained *L. acidophilus* cell with 15% glycerol, 5% or 10% starch. The microcapsules with 10% starch had a relatively rounded, smooth surface; the bacteria density reached 1.04×10^{10} cfu/g and water content were 0.032, which were the best results among all formulas. After 3 months of storage at 4°C, those microcapsules still met the requirement of bacteria density and water activity and was capable of producing probiotics material in the pharmaceutical industry.

6. ACKNOWLEDGEMENT

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