The Protective Effect of Microcapsules (Pullulan / Sucrose / Whey Protein) on Vitality of *Lactobacillus bulgaricus* QY-2

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**Abstract.** The purpose of this study was to prepare microencapsules containing *L. bulgaricus* QY2 by using pullulan, sucrose and whey protein as wall material and to evaluate the survival of the encapsulated *L. bulgaricus* QY2. Pullulan, sucrose and whey protein were selected to optimize the compound protective agent, L9 (3³) orthogonal test design was carried out to determine the activity of *L. bulgaricus* QY2. Through the analyses that can reflect the core material for encapsulant protection indicators such as survival rate and vitality of *L. bulgaricus* QY2. When the microcapsule wall material was composed of 2 wt% pullulan, 10 wt% sucrose and 10 wt% whey protein, the survival rate of *L. bulgaricus* QY2 was the highest. In addition, the *L. bulgaricus* QY2 microcapsule combination of pullulan at 1 wt%, sucrose at 10 wt% and whey protein at 10 wt% had the highest activity.

1. Introduction

Microcapsule technology is a kind of new technology with rapid development, and widely used in the world today. Microencapsulation is a micro- “container” or “packaging” that can bury and protect particles or droplets of certain substances so that it has a semi-permeable or sealant nature of the polymer wall shell [1,2]. Microencapsulation refers to the use of polymer polymers or other film forming materials to cover solids, liquids, and even gaseous substances, and to make microcapsules with capsule walls and retain or intercept other material particles through technical means. The shape of this microcapsule formed by microencapsulation technology is diverse. It can be a thin film, spherical grape string or an irregular shape. The outer surface of the capsule is smooth and folded [3]. There is no doubt that the selection of embedded wall materials is the most critical part in the study of probiotics microencapsulation, and different embedded wall materials have their own physical and chemical properties and embedding methods. Although a variety of polysaccharide polymers such as carrageenan, starch, guar gum, carboxymethyl cellulose and so on have been reported to be used for the inclusion of probiotics [4,5], but considering the practicality and economy of *Lactobacillus bulgaricus* QY2 inclusion, sodium alginate and protein used as the wall materials were most widely studied at present. There are three main methods of probiotics microencapsulation: spray drying, extrusion and emulsification [6]. Although spray drying is rarely used for the embedding of heat-sensitive bioactive components, probiotics cells can be successfully embedded in the protein wall material through spray drying by controlling the process conditions (air inlet temperature, air outlet temperature and feed speed, etc.) [7,8]. The general process of the extrusion method is to mix the probiotics cell suspension with sodium alginate solution, and then drop it into CaCl₂ solution through a
syringe or atomizer for gel forming. The extrusion method has the advantages of simple operation, low cost and little damage to probiotics. However, the biggest disadvantage of this method is that the yield is low and it is difficult to be industrialized and mass-produced [9]. In addition, the particle size of microcapsules prepared by extrusion method is usually greater than 1 mm [10]. The traditional emulsification emulsion method is to disperse the mixture of sodium alginate and probiotics cells into the oil phase. After the W/O emulsion is formed, the CaCl2 solution is added into the emulsion, the oil phase is removed by phase separation method, and the sodium alginate microcapsule is obtained by filtration from the water phase [11]. Xiao Q et al. found that if the particle size of microcapsules was less than 100 μm, the survival rate of L. bulgaricus QY2 in simulated gastric juice could not be improved [12].

Singh RS reported that pullulan have good membrane properties, strong plasticity and viscosity, soluble in water, non-toxic and harmless, colorless and odorless, and have been widely used in the fields of pharmaceutical and food chemicals and petroleum. Pullulan meets the requirements of the coating agent Matrix, solubility is not affected by pH, it can prevent the oxidation of the core material, and it can form membrane embedded probiotics compared with other polysaccharides, and it has a strong protective effect on the activity of the species. Chen C et al. reported that after mixing pullulan and probiotics into a membrane and storing them at 4 °C for 2 months, the survival rate of probiotics was about 70-80 % [14]. It can be seen that pullulan has a good protective effect on probiotics. It is hoped that it will have important social and economic significance to meet the demand of Yoghurt market, improve the production level of Yoghurt fermentation agent and improve the competitiveness of independent property right fermentation agent.

2. Materials and methods

2.1. Materials

L. bulgaricus QY2: Research Center of Microecological Engineering Technology, Qiqihar Medical University to provide the strain. MRS culture medium was obtained from Research Centre of Microecological Engineering Technology, Qiqihar Medical University, Heilongjiang province, the People’s Republic of China. Pullulan was purchased from Shanghai Aladdin Biochemical Technology Co., Ltd. Demineralized Whey Powder D70 (DWP, 2.5% of moisture, 12.0% of total protein, 1.5% of fat, 3.0% ash, and 78 - 84% of lactose) was obtained from Alpavít Käserei Champignon Hofmeister GmbH & Co. (Lauben, Germany). Sucrose was purchased from Shanghai Aladdin Biochemical Technology Co., Ltd.

2.2. Methods

2.2.1. Culture of the tested strain: L. bulgaricus QY2 was cultivated twice at 37°C in L-MRS medium in a row under anaerobic conditions. The cultures (30 mL) were centrifuged (6000 r/min, 5min) to collecting cell precipitates, and then L. bulgaricus QY2 were washed twice with saline solution.

2.2.2. Preparation of seed protection liquid: Using pullulan, sucrose and whey protein as wall material and L. bulgaricus QY2 as core material, microcapsules were prepared by the method of freeze-drying, and the protective effects of microcapsules based on pullulan, whey protein and sucrose which were optimized by orthogonal experimental design. Among them, the survival rate and the vitality of L. bulgaricus QY2 were evaluated by performing viable cell counts immediately after the beads’ preparation.

2.2.3. Covering the count of L. bulgaricus QY2: The method of covering the L. bulgaricus QY2 is mainly based on Maganha L C et al adding microcapsules to phosphate buffers, slowly shaking the broken microcapsules through high-speed homogenies, slowly shaking the broken liquid 30 min, take a certain amount of liquid, after dilution, it is coated on the MRS agar medium and then the plate is counted after 48 h culture at 37 °C under anaerobic conditions [15]. The centrifuge tube containing freeze-dried L. bulgaricus QY2 powder was placed at room
temperature for 30 min. After the temperature returned to room temperature, sterile water of the same volume as the medium was added and oscillated to fully mix the bacteria. Take a number of test tube containing 4.5 mL of aseptic water, adding 0.5 mL of mixed freeze-dried bacteria powder solution, oscillating to fully mix it, and then dilute the bacteria solution to 10^6 CFU/mL, from which drawing 50 μL to 4 mL L-MRS medium, cultivate 7 h to detect the absorption value at a wavelength of 600 nm, each group of 3 parallel. Calculating \textit{L. bulgaricus} QY2 survival rate according to equation 1:

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\text{\textit{L. bulgaricus} QY2 survival rate (\%)} = \left( \frac{A_2}{A_1} \right) \times 100
\]

\(A_1\) —— \textit{L. bulgaricus} QY2 liquid OD\textsubscript{600} before freeze-drying;
\(A_2\) —— \textit{L. bulgaricus} QY2 liquid OD\textsubscript{600} after freeze-drying;
OD\textsubscript{600} —— The absorption value at a wavelength of 600 nm;

3. Results and Discussion

3.1. Orthogonal test for different parietal content

According to the different protective effects of the compound protectant, the cells can be protected together in freeze drying. The best protective effect can be achieved only when the concentration ratio of each protective agent in the compound protective agent reaches the coordination. Therefore, on the basis of the above research results, it is proposed to combine different types of protective agents to make them play their respective roles. Sucrose, whey protein and pullulan were selected to optimize the compound protective agent, and the analysis of the experimental results was shown in Table 1. L9 (3^3) orthogonal test design was used to determine the activity of \textit{L. bulgaricus} QY2 by using pullulan, whey protein and sucrose as three factors. Through analysis, the optimal ratio of each factor to the activity of \textit{L. bulgaricus} QY2 was found.

| Text | Pullulan (wt%) | Sucrose (wt%) | whey protein (wt%) | Bacterial vitality (×10^9) | Survival rate (%) |
|------|---------------|--------------|-------------------|---------------------------|-------------------|
| 1    | 0             | 0            | 0                 | 0.21±0.03                 | 3.40±0.83         |
| 2    | 0             | 5            | 5                 | 19.56±2.26                | 41.24±5.64        |
| 3    | 0             | 10           | 5                 | 9.57±1.25                 | 36.80±4.32        |
| 4    | 1             | 0            | 5                 | 23.07±2.37                | 22.00±3.27        |
| 5    | 1             | 5            | 10                | 21.42±2.45                | 41.36±4.21        |
| 6    | 1             | 10           | 0                 | 29.10±3.56                | 37.04±3.21        |
| 7    | 2             | 0            | 10                | 13.11±2.11                | 36.79±2.87        |
| 8    | 2             | 5            | 0                 | 4.06±0.98                 | 18.80±1.96        |
| 9    | 2             | 10           | 5                 | 11.22±1.12                | 34.27±2.45        |

*Data were expressed as mean ± standard deviation, n=3.

According to variance analysis, there is no significant difference in the average number of bacteria at different levels of each factor. In this case, the level with a larger average can be selected from the table is A\textsubscript{2}B\textsubscript{3}C\textsubscript{2}.

Description of statistical methods: SAS 9.2 software was used for statistical analysis. Survival rate and viability were described by \( \bar{x} \pm s \), and analysis of variance (ANOVA) with orthogonal design was used for comparison between groups (The results were shown in Table 2).
Table 2. Mean and standard deviation of various levels of different factors (\( \bar{x} \pm s \)).

| Content (%) | Pullulan  | Content (%) | Sucrose  | Content (%) | Whey protein |
|-------------|-----------|-------------|----------|-------------|--------------|
| 0           | 0.038±0.028 | 0           | 0.207±0.167 | 0           | 0.264±0.199  |
| 1           | 0.335±0.102 | 5           | 0.271±0.225 | 5           | 0.192±0.167  |
| 2           | 0.366±0.023 | 10          | 0.260±0.167 | 10          | 0.283±0.188  |

Table 3. Results of ANOVA for survival rate.

| Factors       | Degrees of freedom | Sum of squares from mean (SS) | The mean square (MS) | \( F \)  | \( P \) |
|---------------|---------------------|------------------------------|---------------------|---------|-------|
| Pullulan      | 2                   | 0.1966                       | 0.0983              | 87.52   | 0.0113|
| Sucrose       | 2                   | 0.0070                       | 0.0035              | 3.13    | 0.2419|
| Whey protein  | 2                   | 0.0140                       | 0.0070              | 6.22    | 0.1385|

It’s shown that the survival rate was statistically significant only among the pullulan groups at \( \alpha=0.05 \) (\( F=87.52, P=0.0113 \)). Combined with Table 1, the combination of pullulan at 2 wt%, sucrose at 10 wt% and whey protein at 10 wt% had the highest survival rate.

Table 4. Mean and standard deviation of each level of different factors (\( \bar{x} \pm s \)).

| Content (%) | Pullulan  | Content (%) | Sucrose  | Content (%) | Whey protein |
|-------------|-----------|-------------|----------|-------------|--------------|
| 0           | 7.409±2.912 | 0           | 13.426±9.490 | 0           | 14.419±3.059 |
| 1           | 25.863±3.30  | 5           | 14.680±9.331 | 5           | 14.283±7.725 |
| 2           | 11.463±1.53  | 10          | 16.630±10.831| 10          | 16.033±8.320 |

Table 5. Results of ANOVA for survival rate.

| Factors       | Degrees of freedom | Sum of squares from mean (SS) | The mean square (MS) | \( F \)  | \( P \) |
|---------------|---------------------|------------------------------|---------------------|---------|-------|
| Pullulan      | 2                   | 564.3450                     | 282.1725            | 29.94   | 0.0323|
| Sucrose       | 2                   | 15.6406                      | 7.8203              | 0.83    | 0.5465|
| Whey protein  | 2                   | 5.6860                       | 2.8430              | 0.30    | 0.7682|

The results of statistical analysis (Table 4 and Table 5) showed that the difference of \( L. \) bulgaricus QY2 activity was statistically significant only among pullulan groups (\( F=29.94, P=0.0323 \)) according to the test level of \( \alpha=0.05 \). Combined with Table 3, the combination of pullulan at 1 wt%, sucrose at 10 wt% and whey protein at 10 wt% was used to achieve the highest \( L. \) bulgaricus QY2 activity.

4. Conclusion
In this study, it shown that pullulan/sucrose/whey protein microcapsules have good protective effect on \( L. \) bulgaricus QY2 in this study. The microcapsules have good physical properties can improve the survival rate and the vitality of \( L. \) bulgaricus QY2. Therefore, this microcapsule is a good new material for encapsulating and protecting \( L. \) bulgaricus QY2.

Acknowledgments
This work was supported by grants from the Fund Projects of Qiqihar Medical University (Grants No. QY2014Q-01)
5. Reference

[1] Mehta A M. Microencapsulation: US 1989.

[2] Jackson L S, Lee K. Microencapsulation and the food industry[J]. Lebensmittel-Wissenschaft + Technologie, 1991, 24(4):289-297.

[3] Madene A, Jacquot M, Scher J, et al. Flavour encapsulation and controlled release – a review[J]. International Journal of Food Science & Technology, 2010, 41(1):1-21.

[4] Anal A K, Singh H. Recent advances in microencapsulation of probiotics for industrial applications and targeted delivery[J]. Trends Food Sci Technol, 2007, 18(5):240-251.

[5] Mortazavian A, Razavi S H, Ehsani M R, et al. Principles and methods of microencapsulation of probiotic microorganisms[J]. Iranian Journal of Biotechnology, 2007, 5(1):1-18.

[6] Hansen L T, Allan-Wojtas P M, Jin Y L, et al. Survival of Ca-alginate microencapsulated Lactobacillus bulgaricus spp. in milk and simulated gastrointestinal conditions[J]. Food Microbiology, 2002, 19(1):35-45.

[7] Sheu T Y, Marshall R T. Microentrapment of Lactobacilli in Calcium Alginate Gels[J]. Journal of Food Science, 2010, 58(3):557-561.

[8] Singh R S, Saini G K, Kennedy J F. Pullulan: Microbial sources, production and applications[J]. Carbohydrate Polymers, 2008, 73(4):515-531.

[9] Kannmani P, Lim S T. Development and characterization of novel probiotic-residing pullulan/starch edible films[J]. Food Chemistry, 2013, 141(2):1041-1049.

[10] Trovatti E, Fernandes S C M, Rubatat L, et al. Pullulan-nanofibrillated cellulose composite films with improved thermal and mechanical properties[J]. Composites Science & Technology, 2012, 72(13):1556-1561.

[11] Wu J, Zhong F, Li Y, et al. Preparation and characterization of pullulan–chitosan and pullulan–carboxymethyl chitosan blended films[J]. Food Hydrocolloids, 2013, 30(1):82-91.

[12] Xiao Q, Tong Q, Lim L T. Pullulan-sodium alginate based edible films: Rheological properties of film forming solutions[J]. Carbohydrate Polymers, 2012, 87(2):1689-1695.

[13] Bakker-Zierikzee A M. Prebiotics and probiotics in infant nutrition[J]. Pediatr Med Chir, 2005, 29(2):69-83.

[14] Chen C C, Walker W A. Probiotics and Prebiotics: Role in Clinical Disease States[J]. Advances in Pediatrics, 2005, 52(none):77-113.

[15] Maganha L C, Rosim R E, Corassin C H, et al. Viability of probiotic bacteria in fermented skim milk produced with different levels of milk powder and sugar[J]. International Journal of Dairy Technology, 2014, 67(1):89-94.