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# Formulation Technology of a Probiotic *(Zymomonas mobilis)* in Gelatinous Capsules

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**SUMMARY. This study aimed the obtainment of a probiotic in the pharmaceutical form of capsules by using fermented *Zymomonas mobilis* standardized as raw matter. Lyophilization of the fermented *Z. mobilis* with a cryoprotective solution (10% saccharose, 1% gelatin and 4% colloidal silicon dioxide) resulted on the best formulation obtained. The capsules obtained from that product of lyophilization had uniform weight, satisfactory time of desintegration and dissolution, and low humidity content, both in the laboratory scale and in the transposition to the semi-industrial scale. The technological processes used did not alter *Z. mobilis* cell viability.**

*RESUMEN*. "Tecnología de la Formulación Farmacéutica de un Probiótico *(Zimomonas mobilis)* en Cápsulas de Gelatina Duras". Este estudio tuvo como objetivo la obtención de un probiótico en la forma farmacéutica cápsula utilizando como materia prima *Zimomonas mobilis* fermentado. La liofilización del fermentado de *Z. mobilis* en una solución crioprotectora (10% de sacarosa, 1% de gelatina y 4% de dióxido de silicio coloidal) resultó ser la mejor formulación alcanzada. Las cápsulas obtenidas a partir de este producto liofilizado presentaron uniformidad de masa, tiempo de disgregación y disolución satisfactorios, además de un bajo porcentaje de humedad en el lote a escala de laboratorio, al igual que para el lote a escala semi-industrial. El proceso tecnológico utilizado no alteró la viabilidad de las células de *Z. mobilis*.

## INTRODUCTION

A number of definitions of the term 'probiotic' have been used over the years but the one derived by the Food and Agriculture Organization of the United Nations - World Health Organization and endosed by the International Scientific Association for Probiotics and Prebiotics best exemplifies the breadth and scope of probiotics as they are known today as "live microorganisms, which when administered in adequate amounts, confer a health benefit on the host" and prebiotics as "nondigestible food ingredients that may beneficially affect the host by selectively stimulating the growth and/or the activity of a limited number of bacteria in the colon" [1].

The immune response can be non-specifically stimulated by a number of agents. One of these, *Zymomonas mobilis,* has already been studied, and its antagonistic effects against bacteria, fungi and protozoa are known. Although *Z. mobilis* has been used in several biological systems, little is known about its action on helminthic infections [2].

Those medicinal properties of *Z. mobilis* motivated the commercial production of pharmaceutical products from that probiotic. The development of such products involved the process of lyophilization of fermented *Z. mobilis* for the obtainment of a stable powder, which was used as raw material for the production of gelatinous capsules.

KEY WORDS: Capsules, Fermentation, Lyophilization, Probiotics, *Zymomonas mobilis*.
*PALABRAS-CLAVE:* Cápsulas, Fermentación, Liofilización, Prebiótico, *Zimomonas mobilis*.

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## MATERIAL AND METHODS

The raw material in this study comprised fermented *Z. mobilis,* colloidal silicon dioxide (Aerosil), gelatin, and K-certified saccharose. Fermented *Z. mobilis* was obtained through the standardized fermentative process in the SCHREDER medium (saccharose, 20.0 g/L; yeast extract, 1.0 g/L; $K_2HPO_4$, 1.0 g/L; $MgSO_2.7H_2O$, 0.5 g/L; $(NH_4)_2SO_4$, 1.0 g/L; pH, 7.2), incubated at 35 °C for 48 h.

### *Obtaining the product of lyophilization*

Three different methods of lyophilization were performed. The first method comprised the fermentation of *Z. mobilis* for 48 h. The product of fermentation was frozen in a solution of glycolic alcohol at -40 °C, being then lyophilized (HETO FD3) for 72 h and no cryoprotective solution was used. This batch was obtained on a work scale and denominated Laboratory Lyophilization Batch 1 (LLB 1).

The second method comprised a previous centrifugation of the 48 h fermented *Z. mobilis* at 4500 rpm, for 10 min. The supernatant and the sediment (cell mass) were resuspended in a cryoprotective solution of 10% saccharose and 1% gelatin. The preparation was frozen at -24 °C for 24 h, being then lyophilized at -40 °C for 48 h (LLB 2).

The third method comprised lyophilization performed according to the previously described method; however, colloidal silicon dioxide was added to the cryoprotective solution. Cryoprotective solutions were used with fixed concentrations of 10% saccharose and 1% gelatin, and varied concentrations of colloidal silicon dioxide of 1.5% (LLB 3), 3% (LLB 4), 5% (LLB 5), and 4% (LLB 6) for 24 h.

The definitive formulation of the cryoprotective solution was defined as 10% saccharose, 1% gelatin, and 4% colloidal silicon dioxide (LLB 6). This batch was repeated 4 times, originating the Laboratory Lyophilization Batches (LLB) 7 to 10.

### *Controls performed in the product of lyophilization*

For optimizing the lyophilization methods, the following parameters were assessed: cell viability, aspect, color; efficiency, and time of lyophilization for all lyophilization batches obtained.

The tests for quality control of the products of lyophilization of the laboratory batches 6 to 10 were performed to assure the reproducibility of the technical process. The following parameters were assessed: organoleptic characteristics; cell viability, efficiency, flowing off time, resting angle, apparent volume and density, compact volume and density, Carr index, Hausner index, particle size, and humidity.

Cell viability of the product of lyophilization was assessed through the gross observation of the growth of *Z. mobilis* colonies. The product of lyophilization was reconstituted in sterilized distilled water and spread on semisolid SSDL medium (glucose, 20.0 g/L; yeast extract, 5.0 g/L; agar, 12 g/L; pH = 6.5). The plaques were incubated at 35 °C for 48 h.

The resting angle ($\alpha$) was calculated according to the equation: $tg\ \alpha = h/r$ and the apparent volume and density were determined according to the following equation: $d_{ap} = g/V_{ap}$ (g/mL). The compacted density (dc), Carr index, and Hausner index were calculated according to the following equations: $d_c = g/V_c$ (g/mL), Carr (CI) = $d_c - d_{ap} / d_c$ and Hausner (HI) = $d_c / d_{ap}$ [3,4,5,6].

The analysis of the granules was performed by sieving 30 g of the product of lyophilization with mechanical agitation for 15 min. The residual humidity was assessed by using the METTLER LP12 scale (Infrared).

### *Preparation of Capsules*

Capsules were obtained from the product of lyophilization whose optimal formulation was as follows: concentrate of cells in a cryoprotective solution of 10% saccharose, 1% gelatin, and 4% colloidal silicon dioxide. Four laboratory batches of capsules (Laboratory Capsules Batch - LCB 1 to 4) were produced by using the TEPRON manual encapsulator, and another batch was produced in semi-industrial scale, and denominated Semi-Industrial Capsules Batch 1 (SICB 1) in the ERLY semi-automate encapsulator. The batches were obtained by using number-2 capsules of beige and brown color, which were kept in opaque plastic containers away from heat, light and humidity.

### *Controls performed in the capsules*

The mean weight, time of desintegration, humidity content, and cell viability of the capsules were determined after the preparation of each of the 5 batches obtained (LCB 1-4 and SICB 1). The mean weight, standard deviation and coefficient of variation were calculated according to the twentieth nineth edition of the United State Pharmacopeia [7].

The desintegration time was determined by two immersion fluids which were water and

acid medium. After 30 min, either one of the capsules were completely desintegrated or only a few insoluble fragments of soft consistency remained.

For determining the humidity content, 5 capsules from each batch were kept at 105 °C for 15 min. Cell viability was assessed by means of gross and microscopic observations of the growth of the *Z. mobilis* colonies. The powder contained inside 5 capsules was reconstituted in sterilized distilled water and spread on the semisolid SSDL medium. The plaques were then incubated at 35 °C for 48 h.

## RESULTS AND DISCUSSION

### *Products of lyophilization*

The product of the first method of lyophilization (LLB 1) was porous, yellow and highly hygroscopic. After performing the cell viability test, no viable cell of *Z. mobilis* was observed. This process required 72 h until complete drying of the product was obtained.

The product of lyophilization of LLB 2, with the cryoprotective solution of 10% saccharose and 1% gelatin, had also a porous aspect, was white and very hygroscopic, but the *Z. mobilis* cells remained viable. The cryoprotective solution provided a 24-h reduction in the lyophilization process as compared with the first batch. This second batch also had a greater mass efficiency as compared with the first one. In the presence of humidity, that product of lyophilization lost its porous aspect and acquired a hard gelatinous aspect.

The product of lyophilization obtained with the cryoprotective solution of 10% saccharose, 1% gelatin and 1.5% colloidal silicon dioxide (LLB 3) had a porous aspect, white color and marked hygroscopicity, but maintained cell viability. With the incorporation of colloidal silicon dioxide in the cryoprotective solution, a 48-h reduction in the lyophilization process was observed as compared with that in the first method, but efficiency was maintained.

The cryoprotective solution of saccharose and gelatin provided protection to the microorganism from mechanical and chemical damages during the lyophilization process, because, after its use, *Z. mobilis* cell viability was maintained in the product obtained.

In the LLB 4, where the concentration of colloidal silicon dioxide in the cryoprotective solution was 3%, the product of lyophilization was a white powder that still absorbed humidity during a short exposure, acquiring a hard gelatinous aspect, which caused the formation of compact lumps.

The products of lyophilization obtained from the cryoprotective solution at concentrations of colloidal silicon dioxide of 5 and 4% (LLB 5 and LLB 6, respectively) were a stable powder with a lyophilization time of 24 h. The 4% concentration (LLB 6) was chosen, because the addition of colloidal silicon dioxide at that concentration as a drying adjuvant increased stability and maintained *Z. mobilis* viability. Based on the choice of the best formulation (LLB 6), 4 batches were produced (LLB 7-10), where the reproducibility of the process was confirmed.

Table 1 shows the values obtained for the resting angle and flowing off time referring to the LLB 6 to 10. The angles obtained for the different products of lyophilization showed reproducibility of the process, being the resting angle approximately 36° for the 5 batches assessed.

According to Wells & Aulton [4], the resting angles between 30 and 40° indicate satisfactory properties of fluidity, which can be improved with the addition of a sliding agent. Periods of time shorter than 10 seconds for the passage of the powder or granules in a normalized funnel are considered satisfactory, denoting good fluidity. Therefore, the values found in the batches define an adequate flowing off time for feeding the machines that fill the capsules.

Table 1 shows the apparent volume and density referring to the batches 6 to 10. Based on the data obtained, the 5 batches of products of lyophilization have the same interparticular porosity.

The Carr index values found for the batches studied ranged from 10.71 to 14.25, indicating, therefore, excellent flow properties, according to the relation of fluidity and compressibility [3,4].

Similarly, calculating the Hausner index (HI), we obtained values between 1.12 and 1.16, where those below 1.25 also indicate, by definition, good fluidity. This signals garanties good weight and volume conditions in filling the encapsulation chambers, which was also confirmed by the reproducibility of the values of compacted densities.

The values of residual humidity was around 1%. This reveals that the process of lyophilization provided good drying of the material.

The use of silicon dioxide as a drier has also contributed to the low residual humidity, in addition to improving the fluidity properties of the powder necessary to the accurate measurement of the volumetric filling of the capsules. It acted

| Parameters | LLB 1 | LLB 2 | LLB 3 | LLB 4 | LLB 5 | LLB 6 | LLB 7 | LLB 8 | LLB 9 | LLB 10 |
|---|---|---|---|---|---|---|---|---|---|---|
| Cell viability | nonviable | viable | viable | viable | viable | viable | viable | viable | viable | - |
| Aspect | porous | porous | porous | powder/ hard gel | powder | powder | powder | powder | powder | powder |
| Time of lyophilization (h) | 72 | 48 | 24 | 24 | 24 | 24 | 24 | 24 | 24 | 24 |
| Efficiency (g) | 2.85 | 34.61 | 39.95 | 41.58 | 42.26 | 40.81 | 36.17 | 47.28 | 38.04 | 105.03 |
| Color | yellow | white | white | white | white | white | white | white | white | white |
| Odor | - | - | - | - | - | Char. | Char. | Char. | Char. | Char. |
| Taste | - | - | - | - | - | sweet | sweet | sweet | sweet | sweet |
| Flowing off (g/s) | - | - | - | - | - | 5.92 | 5.76 | 6.14 | 6.12 | 6.02 |
| Resting angle (°) | - | - | - | - | - | 35.34 | 35.09 | 36.37 | 36.64 | 35.28 |
| Compaction tests | | | | | | | | | | |
| $V_{ap}$ (mL) | - | - | - | - | - | 92.31 | 91.80 | 92.48 | 92.10 | 92.84 |
| $V_0$ (mL) | - | - | - | - | - | 82.41 | 78.90 | 81.08 | 81.30 | 81.74 |
| $D_{ap}$ (g/mL) | - | - | - | - | - | 0.325 | 0.326 | 0.324 | 0.326 | 0.323 |
| $D_0$ (g/mL) | - | - | - | - | - | 0.364 | 0.3802 | 0.370 | 0.369 | 0.367 |
| Carr index (CI) | - | - | - | - | - | 10.71 | 14.25 | 12.43 | 11.65 | 11.99 |
| Hausner index (HI) | - | - | - | - | - | 1.12 | 1.16 | 1.14 | 1.13 | 1.13 |
| Residual humidity (%) | - | - | - | - | - | 1.0 | 1.0 | 0.99 | 1.0 | 1.1 |

**Table 1**. Results of the controls performed in the products of lyophilization (Laboratory Lyophilization Batch - LLB). Char.: characteristic.

as a sliding agent. Because of its large surface, colloidal silicon dioxide can adsorb a considerable volume of water.

Regarding the distribution of the particles retained in each of the sieves, their mean size was approximately 267 µm, 93% of them having a mean diameter between 75 and 710 µm, and 65% of them having a mean diameter between 75 and 300 µm. These characteristics of the granules are satisfactory for the obtainment of solid pharmaceutical forms.

### *Capsules*

The mean weight of the capsules obtained was below 300 mg, the mean weight variations not exceeding 10%, which is in accordance with the twentieth nineth edition of the United State Pharmacopeia [7].

Because such product has not been specified in any thesis, the cited immersion fluids were assessed in an attempt to predict the time of desintegration of the capsules obtained. All Laboratory Capsules Batches (LCB 1, 2, 4) and the Semi-Industrial Capsules Batches (SICB 1) had humidity contents of 1.84, 1.92, 1.82 and 1.98%, respectively, which are lower than 3%, the ideal limit for maintaining the rheological qualities and stability levels in the post-encapsulated product. The result of capsules control are described on Table 2.

## CONCLUSIONS

The starting point for obtaining capsules of *Z. mobilis* was a product of lyophilization with adequate rheological properties. Maintenance of cell viability is a priority because the technology used for the preparation of a probiotic may affect efficacy, considering that the temperature and pressure applied during the process may alter cell viability.

This study resulted in the optimization of the lyophilization process for the obtainment of a stable powder with low humidity content and good physical properties for the preparation of the capsules. A cryoprotective solution composed of 10% saccharose, 1% gelatin and 4% colloidal silicon dioxide was used in the process.

The accuracy of the dosages in the production of the capsules decisively depends on good fluidity, being influenced by different factors, such as residual humidity, particle size and form, cohesion and adhesion forces, and apparent and compacted density. After assessing powder fluidity through physical tests, *Z. mobilis* capsules with a satisfactory time of desinte-

| Parameters | Specifications | LCB 1 | LCB 2 | LCB 3 | LCB 4 | SICB 1 |
|---|---|---|---|---|---|---|
| Mean weight (mg) | < 300 | 222.7 | 214.5 | 224 | 219 | 240 |
| Coefficient of Variation (%) | <10 | 4.8 | 8.48 | 5.49 | 6.07 | 6.12 |
| Disintegration (min) $H_2O$ | < 30 | 12 | 10 | 9 | 5 | 8 |
| Disintegration (min) HCl 0.1 M | < 30 | 6 | 5 | 6 | 5 | 6 |
| Residual humidity (%) | < 3 | 1.84 | 1.92 | 3.4 | 1.82 | 1.98 |
| Cell viability | viable | viable | viable | viable | viable | viable |

**Table 2**. Results of the controls performed in the *Zymomonas mobilis* capsules.

gration and low humidity content were produced.

The satisfactory physical and chemical conditions of the product of lyophilization observed in obtaining the capsules in the Laboratory Scale were confirmed when transposing to the Semi-industrial scale. This confirms that the powder used had good flowing off and good filling of the encapsulation chambers, providing capsules with weight regularity.

The product obtained in this study is undergoing a stability study so as to obtain further registration in the regulatory agencies. The stability study is being carried out in an acclimatized chamber, and the transposition to industrial batches abides by the guidelines of the Good Manufacturing Practices (GMP) of the Pharmaceutical Laboratory of the State of Pernambuco (LAFEPE)..

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