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OPTIMIZATION OF THE FERMENTATION CONDITIONS AND SURVIVAL OF *Bacillus licheniformis* AS FREEZE-DRIED POWDER FOR ANIMAL PROBIOTIC APPLICATIONS

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Abstract

Preparations containing probiotic bacteria have a beneficial effect on animal health. The probiotics benefits translate into an increased interest in techniques for the preservation of microorganisms. In this study, the viability of Bacillus licheniformis (BL) ATCC 21424 strain, was evaluated in shake flask culture (Erlenmeyer 100 mL on shaking incubator) and batch 7-L stirred bioreactor under submerged fermentation (SMF), respectively. The inoculum was grown in a nutrient medium (37°C, 24 h±2 h, 200 rpm) and the viability was evaluated by 10-fold dilutions. The fermentation process in the bioreactor was examined at 37°C for 24 h under constant agitation (200 rpm). During SMF under controlled pH and oxygen availability, the cell growth rate was measured by optical density (OD 600 nm) at different interval times (6, 12, 18, 22 and 24 h). The maximum specific rate of BL in the exponential phase was calculated 0.524 h-1. When the stationary phase was reached, the OD in SMF increased, which was 2.01 times higher than that in flask culture. Without any cryoprotectant, the cell suspension was subjected to cold shock first and then freeze-dried. The proven survival rate of cells after freeze-drying was 90.65%. The viability of BL powder decreased only by 1.09 log (CFU/mL) vs. SMF, this resistance being also due to Bacillus spp. ability to sporulate. These results convincingly demonstrated that freeze-drying could be used in the preparation of BL ATCC 21424 strain as a lyophilized probiotic product with applicability in animal nutrition.

Key words*: animal nutrition, Bacillus, bioreactor, freeze-drying, probiotics.*

INTRODUCTION

Defined as “live microorganisms”, probiotics confer health benefits to the host when consumed in adequate quantities (FAO/WHO, 2007). Before probiotics utilization (Pandey & Vakil, 2017), culture bacteria must have the capacity to resist the harvest processing conditions (Dumitru et al., 2021). Moreover, the bacterial strains must retain functionality and viability during storage and transference as the lyophilized product (frozen or freeze-dried technique) with suitability for applications (Pandey & Vakil, 2017). Most probiotics sources are microorganisms from Gram-positive bacteria such as *Lactobacillus, Lactococcus, Streptococcus, Enterococcus, Bifidobacterium*, and *Bacillus* species (Pradipta et al., 2019).

It is known that loss of probiotic cell viability (CFIA, 2009) for long-term storage represents a major limitation factor (Weinbreck et al., 2010). Therefore, at the point of consumption, the viability of probiotic bacteria should contain a minimum level of 10^6 CFU/g (Mahmoud et al., 2020), respectively between 10^7-10^9 CFU/g at the time of ingesting to confer beneficial efficacy (Vidhyalakshmi et al., 2009). Further, probiotics must resist during gastrointestinal tract (GIT) passage, especially at low pH and aggressive intestinal fluids (bile salts and pancreatic juice), storage conditions (oxygen, high temperature, pH variations, relative humidity) and antimicrobial substances, which could determine the loss of cells viability (Cha et al., 2012; Dumitru et al., 2019; Dumitru et al., 2023). Instead, the above-mentioned criteria suggest that the selected strains are essential to be safe, viable and metabolically active within the GIT to involve beneficial results on the host. Moreover, these desirable characteristics facilitate the probiotic transition through the gut and enable bacteria

proliferation and colonization (Divisekera et al., 2019).

As a good strategy to improve the viability of probiotics bacteria during processing, the encapsulation process represents an excellent substitute. Conditions optimization to achieve a dehydrated bacterial product with the possibility to restore its viability after rehydration represents the first step to extending the shelf life without changing the composition and undesirable properties that may appear during storage (Bolla et al., 2010). Several encapsulating techniques are used for the lyophilization of probiotics (Guo et al., 2022), but the most relevant are freeze-drying and spray-drying methods (Mahdi et al., 2020). Freeze drying or lyophilization is a drying process that trusts the sublimation of water in samples (Chantorn et al., 2022). It has been affirmed that is one of the most used procedures for the preservation of bacteria and concentrated starter cultures (Bolla et al., 2010). It is known that, during the lyophilization process, bacterial cells must face certain unfavorable conditions such as low temperature and low water activity, which could lead to decreased bacterial viability due to the damage of cell membranes and proteins (Chantorn et al., 2022). Moreover, the effectiveness of this bacteria preservation technique is up to 10 years (Harrison & Pelczar, 1963). Thus, in the drier form, the candidates' bacteria can be more easily utilized. In this context, the authors examined the viability of the *B. licheniformis* strain during the fermentation process and its subsequent exposure to the freeze-drying with the prior verification of the survival rate, in order to administer it as a source of probiotic product in animal feed.

MATERIALS AND METHODS

Bacterial strain, reagents and materials used

Bacillus licheniformis was delivered by American Tissue Culture Collection (ATCC 21424). The culture bacteria was reactivated in Nutrient broth medium (g/L: tryptone 10; meat extract 5.0; sodium chloride 5.0; pH medium 7.2 ± 0.2 before autoclaving), respectively agar medium (Merck) for cultural traits evaluation, followed by incubation in a shaker-incubator, 200 rpm, 37°C for 24 h. The inoculum was analysed by serial dilution (1:10 *v/v*) in 0.85% sterile physiological serum (SPS) for estimated the counts number (CFU/mL) viability (10^{12}-fold dilutions). From selected dilutions (10^{8}, 10^{10}, 10^{12}), 1 mL was well homogenized and spread on the nutrient agar plate. For each dilution, three replicates were done. The strain was preserved at -80°C with 20% sterile glycerol (*v/v*) and can be found in the Collection of National Research Development Institute for Biology and Animal Nutrition Balotești (INCDBNA), Romania, under the code IBNA 80.

Bioreactor Batch and Fermentation Process

The strain was fermented in a bench-top LAMBDA MINIFOR laboratory bioreactor. This model type is easy to handle and all-important cultural conditions can be measured and controlled. The minimum working volume was 2 L of the 7 L capacity of the bioreactor vessel. The inoculum (200 mL with a concentration of 10^{10} CFU/mL) was used as starter culture and expose to submerged fermentation (SMF) at 200 rpm, 37°C for 22 ± 2 h. The fermentation process was fitted with a temperature sensor, a rotation speed control and a pH sensor which maintained the medium constant at 6.5 ± 0.2 by two automatically peristaltic pomp [20% NaOH (*w/v*) and 1 N HCl (*v/v*)]. A peristaltic pump automatically adjusted the pH value by adding 20% NaOH, respectively 1N HCl. From time to time, as an antifoaming Antifoam 204 agent sterilized silicone oil (Sigma-Aldrich) was added as required (0.01%, *v/v*).

Freeze-drying process

The strain was subject to lyophilization procedure included the following steps: strain characterization (cultural, morphological, biochemical), bacterial biomass obtained after cultivation in nutrient broth (37°C, 18-24 h, 200 rpm) which was recovered and washed twice with PBS buffer (centrifugation 5000 rpm/10 min/4°C), freezing the sediment overnight at -20°C. As equipment was used a 4 L bench scale freeze dryer (Alpha 1-4 LSC basic, Martin Christ, Osterode am Harz, Germany). The process was performed at a pressure lower than 1.030 mbar (i.e., 0.110

mbar), with a condenser temperature of - 50°C for 18±2 h.

Determination of viable cell number

B. licheniformis cells was freeze-dried without protective agent. After freeze-drying, the powder strain was resuspended to the volume before freeze-dried (1:10, *w/v*) and rehydrating with PBS buffer solution. The viable cell number was determined immediately. The counts were enumerated as CFU per gram of powder (Log CFU/g).

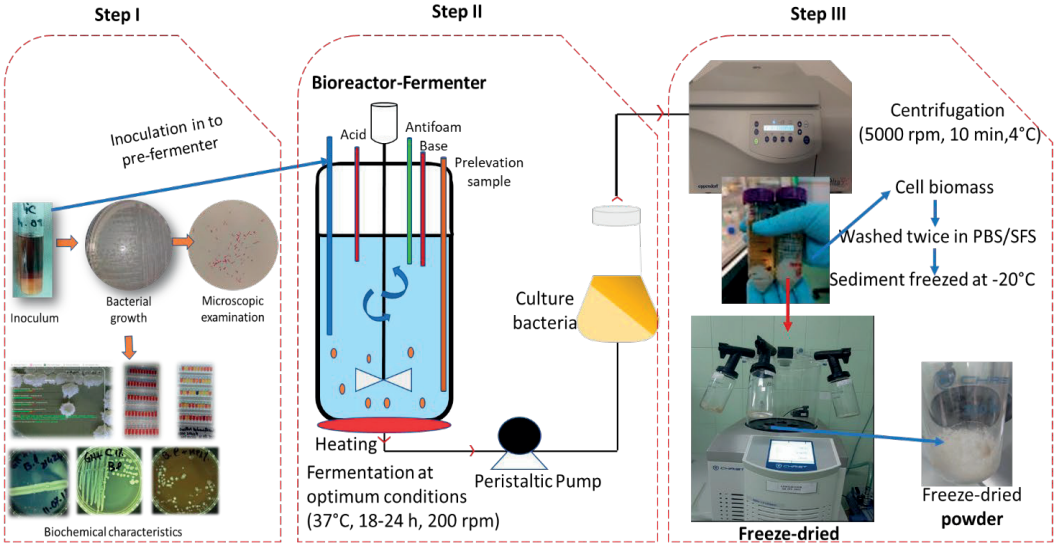

Figure 1. Bioprocess fermentation and lyophilization by freeze-drying

The survival rate was calculated as fallow: Survivability (%) = Log number of viable cells survived after freeze drying (CFU/mL)/Log number of viable cells before freeze-drying (CFU/mL) x 100.

Statistical Analysis

Variance analysis (one-way ANOVA) was used for statistical analysis of the data. All experiments were conducted in triplicate, with three independent measurements. Results are stated as mean values and standard deviation of the mean (SD). The graphics were generated using GraphPad Prism software V. 9.1.2 (Inc., La Jolla, CA, USA).

RESULTS AND DISCUSSIONS

Bacterial strain, reagents and materials used

The taxonomic characterization of *B. licheniformis* strain was detailed in other study (Dumitru et al., 2019a). According to literature data, a considerable group of bacterial probiotics is based on *Bacillus* spp. (*B. licheniformis, B. subtilis, B. cuagulans, B. amyloliquefaciens* etc.). These species are a field of rising scientific interest (Łubkowska et al., 2023). Further, when are added in animal feed, these bacteria provide numerous benefits, facilitate the digestibility, promotion the gut health (He et al., 2020), immune modulation, growth performance, and animal productivity index (Bernardeau et al., 2017; Qiu et al., 2021). Instead, due to the sporulation ability, *Bacillus* spp. form one oval endospore per cell making them to survive to the environmental stress and harsh conditions (Łubkowska et al., 2023). Furthermore, the results presented by Dumitru et al. (2019b) confirmed that the *B. licheniformis* spores presente tolerance and significant survivability in extreme simulated *in vitro* conditions (pH, bile salts, temperature, preservation, and storage). Moreover, the *Bacillus* group are a perfect model of microorganisms (Łubkowska et al., 2023) able to survive stabilization methods used in powder product generation such as freeze-drying (lyophilization), the method that was also used

in the present study, which involves cell dehydration (Goderska, 2012; Kiepś & Dembczyński, 2022).

Bioreactor Batch and Fermentation Process

The fermentation process in a 7 L bioreactor was examined at 37°C for 24 h under constant agitation (200 rpm). During SMF under controlled pH and oxygen availability, the cell growth rate was measured by optical density (OD 600 nm) at different interval times (6, 12, 18, 22 and 24 h). The maximum specific rate of BL in the exponential phase was calculated 0.524 h^{-1}. When the stationary phase was reached, the OD in SMF increased, which was 2.01 times higher than that in flask culture (Figure 2).

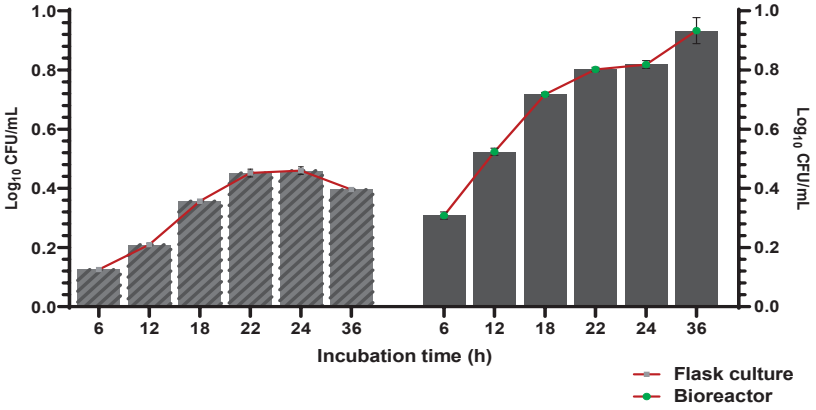

Figure 2. Optical density (OD 600 nm) of *B. licheniformis* strain during different conditions fermentation

This results indicates that SMF fermentation conditions prompted the strain viability *vs*. flask culture during 24 h of incubation.

The harvesting point was reached after 22 h in the bioreactor but with a double growth *vs*. flask cultivation where the pH conditions was non-regulated. Moreover, even from the beginning of the fermentations, the turbidity measurements in both cultivation models were different. At 6 h, the strain registered a point of 0.308 in SMF compared with flask where OD 600 nm was noted 0.126. After 36 h of incubation in the same conditions (37°C, 200 rpm), the cell cultivated without pH regulation established 11.00 Log_{10} vs. 11.70 Log_{10} in SMF, where the pH was 6.8.

Similar to Haindi et al. (2020), our culture strain under controlling acidification conditions began to present a smaller increase in turbidity as compared with the flask culture.

The controller pH, speed agitation (200 rpm), temperature (37°C) hold constant during entire fermentation in the bioreactor. In addition, it can be affirmed that, the pH control during SMF cultivation has a significant influence on strain growth rate.

Freeze-drying process

The experiment was designed to gain information on the cell survivability of a spore strain to produce viable probiotic powder. Without no protectant, the cell suspension was subjected to cold shock first (-20°C) and then freeze-dried for the viable cell number. The proven survival rate of cells after freeze-drying was 90.65%. The viability of *B. licheniformis* powder decreased only by 1.09 log (CFU/mL) *vs*. SMF, this resistance being also due to *Bacillus* spp. ability to sporulate. Further, as can be observed in Figure 3, *B. licheniformis* powder registered a decrease in survivability with 9.35% comparted to the fresh culture where cell number was 5 x 10^{11} CFU/mL.

According to literature data, a product containing probiotic organisms is more efficiently if it contains a number of viable cells higher than 10^6-10^8 CFU/g (Champagne et al., 2011; Dumitru et al., 2023). Insteed, more frequently, the probiotic bacteria used in animal nutrition are included in the form of dried biomass (Kieps & Dembczyński, 2022). Probiotic preparation in solid form, as powder, involve a strong stability and can be stored for

a long period time comparatively than liquide suspensions (Kieps & Dembczyński, 2022). Regarding the drying method, freeze drying or lyophilization presents a multitude of advantages due to the maximisation and extend the viability of probiotic cultures. However, the method is very sensitive and damaging factors in drying the microorganisms must be considered. There are many studies which reported losses of strains viability during freezing technique (Chen et al., 2020; Silva-Carvalho et al., 2020; Luangthongkam et al., 2021). Comparatively with other genus, *Bacillus* group had the capacity to sporulate and the spore-ability involved high resistance to harvest environmental conditions, making this genus a good and strong candidate for developing efficent and stable probiotics products (Mari et al., 2014; Gotor-Villa et al., 2017).

The strain viability

The results on *B. licheniformis* strain growth after SMF fermentation and freeze-drying process was presented in Figure 3. As can be observed, the freeze-drying process decline the strain viability by 1.25% *vs*. SMF.

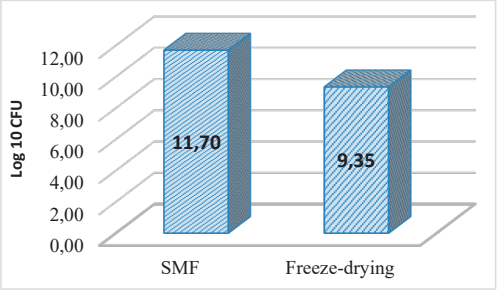

Figure 3. Effect of freeze-drying on *B. licheniformis* strain viability

In this study, satisfactory results were obtained regarding the lyophilization of the bacterial culture without the addition of cryoprotectant. If a decrease in cell viability was observed after freezing compared to SMF fermentation, the differences were not significant and the number of cells recorded was satisfactory.

Obtaining poor results in viability was probably caused by certain parameters such as the time of the drying process, which if it is too fast, the internal water can migrate outside the cell, and the frozen water inside the cell, ultimately leading to the loss of viability (Savedboworn et al., 2019).

Formulations can be completed through different methods including liquid and dry preparations (Gotor-Villa et al., 2017). Compared to liquid forms, generally, dried products, in our case obtained by freeze-drying are more feasible due to their ability to maintain the viability for a long period time of storage. In general, storage at 4°C determined the highest degree of cell viability for bacteria formulated as liquide cultures, but the shelf-life can be short at ambient or higher temperatures (Gotor-Villa et al., 2017).

Frequently, dried products involve lower viability rates because of thermal and dehydration stress found during the drying process (Melin et al., 2007). Besides, genus *Bacillus* is considered very amenable to drying because of its ability of spore production, which provides heat resistance (Yánez-Mendizábal et al. 2012). According to our results, supernatant medium without protectants can be used as medium for preserving *B. licheniformis* ATCC 21424 strain due to the number of living cells registered in the powder form after the freeze-drying method was applied. In addition, it can be stated that the selective culture medium retains, as much as possible, the nutrients necessary for the metabolism of the microorganism and the lyophilization process applied, without determining significant changes in the survival rate of the strain.

CONCLUSIONS

In conclusion, we could affirm that the *B. licheniformis* strain could be satisfactorily formulated with the freeze-drying technique. Nevertheless, this work presented and discussed the fact that the freeze-drying technique could be used in the preparation of the present strain, ensuring efficacy and stability as a lyophilized probiotic product with applicability in animal nutrition.

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