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SHORT COMMUNICATION

Impact of oxygen and humidity on storage of freeze-dried *Lactobacillus gasseri* in relation to water activity and viability

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ABSTRACT

Lactic acid bacteria have shown positive health effects and are widely distributed as dry products. They are widespread applied in functional dairy products, dietary supplements and as pharmaceutical products. Freeze-drying is a process of removing water by sublimation and is one of the most effective preservation technology. The aim of the present study was to establish impact of oxygen and humidity on freeze-dried *Lactobacillus gasseri* during the preparation of the powder and storage time. Lyophilisate was rubbed in aerobic and anaerobic condition with low humidity. Survival test and water activity was investigated after freeze-drying and storage in two different temperatures. The preservation of bacteria was improved when after freeze-drying process lyophilisate have not contact with oxygen and humidity. Survival rates of bacteria was the highest during storage at anaerobic condition both at 4-6 °C and 25 °C. The results shown that it is sufficient to crush of freeze-dried and packaged under anaerobic conditions to achieve higher survival of bacteria.

***Keywords*:** freeze-drying, probiotics, water activity

1. INTRODUCTION

Lactobacillus gasseri is anaerobic, gram-positive bacteria, classified as lactic acid bacteria (LAB). One interesting thing is that these bacteria produce hydrogen peroxide and also have high adhesion properties which lead to biofilm formation. These properties make it a desirable included in pharmaceutical probiotic products [1-10]. LAB are commonly used in dairy, meat products and as a dietary supplements. They may be added in liquid, spray-dried, frozen or lyophilised form. Immobilization methods have been reported as possibilities that can provide protection for sensitive cultures from high oxygen levels, manufacture, storage, freezing, and during transit through the human gastrointestinal tract [6-10]. Freeze-drying (lyophilisation) as one of immobilization method guarantees long-term delivery of stable products [2].

Freeze-drying is one of the commonly used technology for long time preservation of bacteria. The survival of dried bacteria is dependent on many factors such as initial cell concentration, cryoprotectants and drying conditions, etc. [3]. One of negative effects on the survival of dried bacteria that has been observed is oxygen and humidity [3,5]. Dried bacteria immediately after freeze-drying process could absorb oxygen and moisture from the air which could have affect on the survival rate of bacteria during storage. Many studies have been observed effects of oxygen on the survival of freeze-dried bacteria indicating that the hypothesis of a connection between oxidation processes and viability losses in dried bacteria is pertinent [5].

2. MATERIALS AND METHODS

2. 1. Materials and methods

Production of lyophilised

Lactobacillus gasseri was obtained from the collection of the IBSS BIOMED S.A. (Cracow, Poland). Cultures were grown 24 h in modified MRS-broth at 37 °C under anaerobic conditions to stationary phase by using AnaeroGen™ (Oxoid Limited, UK). The modified MRS mediums were prepared according to IBSS BIOMED S.A. protocol. The cells were harvested by centrifugation (5000 x *g*, 10 min, 4 °C), washed with sterile phosphate buffered saline and were centrifuged second time. *L. gasseri* strains were freeze-dried in media contained 80 g/L skim milk and 60 g/L sucrose. The samples were freeze-dried for 72h in freeze-dryer CHRIST® BETA.

For freeze-drying process the bacteria with protective medium were placed in bottles with permeable cap, which after freeze-drying process by moving top shelf were closed hermetic bottles. The samples were transferred to shelf at a room temperature and cooled to -35 °C at 0.180 mbar. The shelf temperature was increased at 20 °C to initiate the sublimation phase. After sublimation phase the shelf temperature was increased to 25 °C to initiate desorption phase. After freeze-drying process samples were closed in freeze-dryer under vacuum condition. One part of samples was transferred to anaerobic chamber with relative humidity (<10%), second part to aerobic condition. A lyophilized powder was obtained under two different conditions using the same sieve of size 0.5 mm.

Storage experiment

The freeze-dried samples of *Lactobacillus gasseri* obtained in an anaerobic chamber and in aerobic condition were stored in two temperatures: 4-6 °C in a fridge and 25 °C in a climatic chamber. The glass bottle with samples were packed in aluminium foil which is total barrier for oxygen. The lyophilised samples that were grated in anaerobic condition were open in anaerobic cabinet before survival and water activity tests. For each relative condition two bottles with lyophilised were prepared: the first one was used for survival test, the second one for measuring water activity.

Survival test

The survival rates of bacteria in freeze-dried powder were expressed as CFU/ g (colony forming units per gram of dry material). One survival test was determined after lyophilisation for each condition. The survival rates during storage were evaluated after 1 month and 3 month for each storage condition. Samples of 0.1 g of lyophilisate were added to 100 ml of saline where upon appropriate solutions were prepared. 100 μL of the three selected dilutions were transferred into triple sets of Petri dishes with modified MRS-agar. The samples were incubated under anaerobic atmosphere in desiccators using AnaeroGen^{TM} (Oxoid Limited, UK) at 37 °C for 48h. Oxygen concentration was identified using Anaeronic Indicator (Oxoid Limited, UK). The colonies were counted and expressed as CFU g^{-1}.

Water activity measurement

The water activity is one of the most important factors infusing the stability of dry and dehydrated products during storage [4]. The water activity of the samples was measured at 25 °C using an a_w meter ms1 Set-aw (Novasina, Switzerland). The water activity was measured after storage simultaneously. The few studies have observed that the viability of bacteria decreased when the water activity increased [3,4].

3. RESULTS AND DISCUSSION

Many studies have investigated the effects impact on viability and stability of freeze-dried bacteria, but knowledge about impact of oxygen and humidity for freeze-dried bacteria is still limited. The results of the survival tests have shown that contact of the lyophilisate with oxygen and humidity after freeze-drying had a significant effect on the storage stability (Table 1). The samples grated in anaerobic condition with low humidity had a higher survival rates than grated in aerobic conditions. If taking into account the also storage temperature the best results after 3 months was a sample grated in anaerobic condition and stored in at 25 °C (Fig. 1).

The viability of freeze-dried *Lactobacillus gasseri* depends on the water activity. Increasing water activities decreased the bacteria survival as seen in Table 2. The highest water activity shows samples grated in aerobic condition and storage at 25 °C. The results show also that a_w above 0.200 the viability loss is increased.

Table 1. Viability of *L.gasseri* during storage in different conditions.

Time	Viability (CFU g^{-1})			
	Storage at 4-6 °C		Storage at 25 °C	
	anaerobic	aerobic	anaerobic	aerobic
0 month	$5{,}40\times10^{11}$	$5{,}40\times10^{11}$	$1{,}68\times10^{11}$	$1{,}68\times10^{11}$
1 month	$4{,}75\times10^{10}$	$5{,}40\times10^{9}$	$2{,}40\times10^{11}$	$1{,}03\times10^{11}$
3months	$2{,}26\times10^{11}$	$1{,}55\times10^{9}$	$2{,}60\times10^{9}$	$4{,}50\times10^{8}$

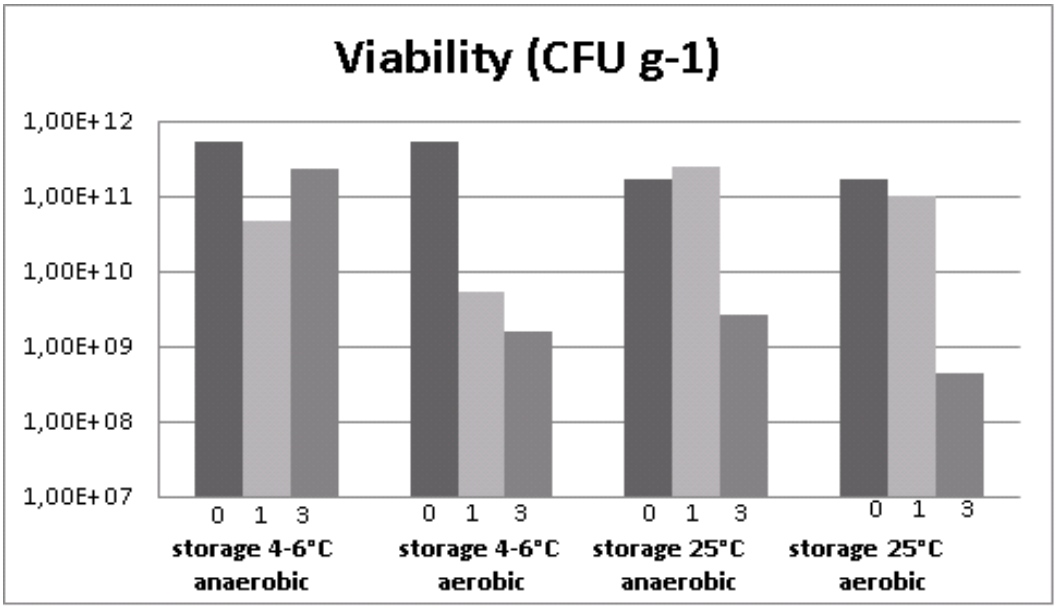

Fig. 1. Graph results of survival tests in different conditions

Table 2. Water activity results during storage.

Time	a_w			
	Storage at 4-6 °C		Storage at 25 °C	
	anaerobic	aerobic	anaerobic	aerobic
0 month	0.041	0.06	0.045	0.157
1 month	0.052	0.064	0.053	0.121
3 months	0.067	0.089	0.053	0.235

The present study has revealed important results on the effects on storage stability of anaerobic bacteria *Lactobacillus gasseri.* The results show that oxygen, humidity, temperature and water activity are significant parameters influence for viability of bacteria *Lactobacillus gasseri*. These parameters are comparatively to control and they should be used in freeze-drying process. These parameters do not increase the cost of production of freeze-dried compared to the fairly expensive cryoprotectants. The use of an anaerobic chamber to grated lyophilisate is additional step not affecting the substantial lengthening of the whole process. Lyophilizer must be equipped bottle closure system under vacuum conditions by raising the top shelf. This allows for the transfer of bottles with lyophilisate to the anaerobic chamber.

Using aluminium foil which is oxygen barrier for packing lyophilisate is additional protection against oxygen and humidity.

4. CONCLUSIONS

1. Oxygen has a strongly negative impact on the viability of lyophilised *L. gasseri* bacteria.
2. The obtained results do not allow to clearly defined the impact of changes in water activity on the viability of freeze-dried *L. gasseri* bacteria
3. To maintain the high rate of freeze-dried *L. gasseri* viability the lyophilisate should be grated in anaerobic conditions and lowered humidity (<10%) and stored at the refrigerator temperature.

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