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Effect of Freeze - Drying on Viability and Probiotic Properties of a Mixture of Probiotic Bacteria

R. M. U. S. K. Rathnayaka
Department of Food Science and Technology, Faculty of Applied Sciences, Sabaragamuwa University of Sri Lanka,
P. O. Box 02, Belihuloya, Sri Lanka
udayarathnayaka@gmail.com

ABSTRACT

The effect of freeze drying on viability and probiotic properties of a probiotic microbial mixture containing Lactobacillus rhamnosus and L. plantarum was studied. Ability of UHT milk, sucrose, sorbitol and trehalose to protect viability and probiotic properties of freeze dried microbial mixture during six month storage at 4 ^{0}C was also studied. Five types of mixed probiotic cultures were prepared as microbial mixture in PBS, UHT milk, UHT milk added with sucrose, UHT milk added with sorbitol and UHT milk added with trehalose. Those were freeze dried and stored at 4 ^{0}C for six months. Immediately after freeze drying and at day 30, 60, 90, 120 and 180 during storage, those microbial mixtures were tested for viability and probiotic properties. Enumeration of samples taken from cultures in MRS agar media and Shigella inhibitory assay was used for the testing of viability and probiotic properties respectively. Better survival of microorganisms after freeze drying was shown in all tested microbial mixtures except the microbial mixture in PBS. Highest microbial survival was shown in the microbial mixture UHT milk. Addition of other cryoprtectant sucrose, sorbitol and trehalose did not improve the microbial survival than UHT milk alone. Hence, addition of those to UHT milk was found ineffective. According to the results of Shigella inhibitory assay, probiotic properties of microbes has not been changed by freeze drying or long term storage. Freeze drying can be used for preservation of tested probiotic cultures in UHT milk without any significant effect to its viability and probiotic properties.

Keywords: *Probiotic cultures, Cryoprotectants, Freeze drying.*

1. INTRODUCTION

Probiotic food products which contain microbial strains with beneficial characters are becoming more and more popular. Consumers are attracted to these products mainly due to high publicity given by the manufacturers on their health benefits. However, these health benefits are depending on the viability of probiotic microbes and maintenance of their probiotic properties in commercial stock cultures and probiotic food products during storage.

It is recommended that probiotic food products should contain at least 10^7 live microorganisms per g or per ml [1]. Hence a commercial probiotic culture should contain high number of viable and active probiotic microbes. As such, large scale production of such cultures in a form suitable for product application by inexpensive method is highly desirable [2].

Different authors have reported successful production of freeze dried and spray dried powders of probiotic microbial cultures of different microbes in different media [3, 4, and 5]. Compared to freeze drying, spray drying is an inexpensive method. It has been estimated that the cost of spray drying is six times lower than the cost of freeze-drying per kilogram of water removed [6]. Further, these spray-dried powders can be transported at a low cost and can be stored in a stable form for prolonged periods [2]. However, due to relatively high temperature used in this method, survival of microbes is comparatively low. Hence, for some microbes freeze drying is more suitable than spray drying [7]. However for some microbes, there is no difference in viability loss between these methods [8].

Although freeze drying has been used to preserve probiotic cultures, there are some are some records on loss of culture viability due to freeze-thaw process [2]. To reduce the loss of viability of probiotic cultures due to freeze drying crypprotectants such as skim milk are commonly used [9, 10]. Further, alternative cryoprotectants such as disaccharides (e.g. sucrose, lactose and trehalose), polyalcohol's (e.g. mannitol and sorbitol), amino acids and proteins [11, 12] also can be used.

The aim of this work was to evaluate the effects of different cryoprotectant used in freeze drying to the survival of mixed probiotic culture and effect of those to the probiotic properties of freeze dried microbial mixture.

2. MATERIALS AND METHODS

2.1 Preparation of Probiotic Microbial Suspensions

Two probiotic bacteria, Lactobacillus rhamnosus and L. plantarum were used in this study. Those were cultured in MRS broth at 37^{0}C for approximately 15 h. The cultures were then harvested by centrifugation at 10000 g for 15 min. and were re-suspended in equal volume of phosphate buffered saline (PBS).

2.2 Preparation of Different Probiotic Microbial Mixtures

Using probiotic microbial suspensions prepared as mentioned above, five different microbial mixtures were prepared. First one was the microbial mixture in PBS which was prepared by mixing 1 ml of each prepared microbial suspension. Second one was the microbial mixture in UHT milk. That was prepared by mixing 1 ml of each microbial suspension followed by centrifugation at 10000 g for 15 min. and re-suspension in 1ml of sterile UHT milk. The third fourth and fifth microbial mixtures were microbial mixtures in UHT milk added with sucrose, sorbitol and trehalose respectively. To prepare those, 1 ml of each microbial suspension were mixed, centrifuged at 10000 g for 15 min. and re-suspended in 1 ml of sterile UHT milk and the added with sucrose, sorbitol and trehalose in 300 mM concentration separately.

2.3 Freeze Drying

Micro tubes containing 200 µl of each prepared probiotic microbial mixtures frozen at -80 ^{0}C for 8 h and then freeze dried for 24 h. The freeze dried cells were stored at 4 ^{0}C up to six months. For enumeration of viable probiotic cells and to test their probiotic properties, samples were taken immediately after freeze drying and different time intervals (after 30 days, 60 days, 90 days, 120 days and 180 days) during the storage. Those were re-hydrated to original volume using distilled water and used for the tests.

2.4 Preparation of Microbial Culture to Test Probiotic Properties

In the present study, Shigella sonnei which has been used previously for testing bactericidal effect of probiotic organisms [13] were used for the same purpose. The bacterium was cultured in tryptone soy broth (TSB) media at 37 ^{0}C for 18 h with shaking. Then the bacterial culture which contained bacterial concentration of 10^9 CFU / ml was centrifuged at 3000 g for 15 min. and bacterial pellet was obtained. Then the pellet was suspended in equal volume of TSB. Ten milliliter of each freeze dried sample taken immediately, 30 days, 60 days, 90 days, 120 days and 180 days after freeze drying was added to the Shigella suspension separately. Then those were incubated with shaking at 37 ^{0}C and enumerated by culturing in Shigella specific media Xylose Lysine Deoxycholate agar (XLD agar) at 37 ^{0}C for 24 h. Control treatments were also performed without freeze dried probiotic cultures at the same conditions. Experiments were conducted with three replicates.

2.5 Enumeration of Probiotic Microbial Cells

Using the samples obtained from freeze dried microbial mixtures, serial dilutions (10^{-1} – 10^{-9}) were prepared in 0.1 % tryptone. Those were placed on MRS agar medium and incubated at 37 ^{0}C for 24 h. number of viable cells was determined as colony forming unit per milliliter (CFU / ml).

3. RESULTS AND DISCUSSION

Ability of a freeze dried mixture of probiotic microorganisms to maintain their viability and probiotic properties during six month storage at 4 ^{0}C was investigated in the present study.

3.1 Effect of Freeze Drying and Storing on Viability

Although the ability to preserve individual microbial strains using freeze drying is well documented, there are only few reports available on preservation of probiotic microbial mixtures using freeze drying [13, 14 and 15]. In the present study, ability of UHT milk and UHT milk added with different cryoprotectants (sucrose, trehalose and sorbitol) to protect the viability of probiotic microbial mixture during storage at 4 ^{0}C for six months after freeze drying was studied.

All freeze dried sample except the microbial mixture in PBS were able to maintain minimum standard level of microbial concentration (10^7 CFU/ml) for probiotic microbial cultures. Higher number of viable count was observed for microbial mixture in UHT milk without added crayoprotectant compared to UHT milk with added different crayoprotectant (table 1). These unexpected results indicate the inability of using cryoprotectant to improve the viability of freeze dried microbial mixtures in UHT milk.

These results are in agreement with some previous similar studies. In one of such study it has been reported the inability of cryoprotectant to improve the viability of freeze dried microbial mixture [13]. In another study it has been reported the inability of addition of sugar to skim milk to improve the viability of Lactobacillus plantarum and Lactobacillus rhumnosus during storage after freeze drying [11].

However, these results are not in agreement with some previous reports on using crayoprotectants to improve the tolerance of microbial cells to freeze drying. In one study it has been reported the ability of sucrose and trehalose to improve the tolerance to freeze drying of Escherichia coli and Bacillus thuringiensis cells [16]. In another study it has reported the ability of sucrose to improve the tolerance of Bifidobacterium animalis to freeze drying without milk based ingredients [17].in another similar study it has been reported the ability of sucrose, sorbitol and trehalose to improve the tolerance of Lactobacillus rhamnosus and L. plantarum for freeze drying [18].

In most of the previous studies sucrose has been identified as a good cryoprotectent which improve the

tolerance of different microbes in freeze drying compared to other cryoprotectants [17, 18]. However, in another study it has been reported a reduction in survival rate of a mixture of lactic acid bacteria and yeast isolated from Kefier when freeze dried using sucrose as a cryoprotectant 13]. As per the results of the present study, no difference was observed in tested three cryoprotectants in their ability to improve tolerance of probiotic microbes tested to the freeze drying

Table 1: Average microbial cell count (CFU / ml) in five prepared and freeze dried mixed cultures during the storage at 4 ^{0}C.

Microbial mixture	Days after freeze drying					
	0	30	60	90	120	180
in PBS	1.84×10^7	3.25×10^6	7.43×10^5	4.28×10^5	4.22×10^4	3.56×10^4
in UHT milk	8.24×10^{11}	2.04×10^{11}	7.38×10^{10}	9.35×10^9	2.45×10^9	6.82×10^8
in UHT milk + Sucrose	7.33×10^{11}	1.11×10^{11}	6.98×10^{10}	7.35×10^9	1.89×10^9	6.32×10^8
in UHT milk + Sorbitol	6.96×10^{11}	1.14×10^{11}	6.33×10^{10}	8.21×10^9	2.11×10^9	5.89×10^8
in UHT milk + Trehalose	7.25×10^{11}	1.32×10^{11}	5.43×10^{10}	7.89×10^9	2.41×10^9	6.22×10^8

*Initial microbial concentration was 2.58×10^{12} CFU/ml

3.2 Effect of Freeze Drying and Storing to the Probiotic Properties

Probiotic cultures should be viable as well as functionally active during long term storage to have their expected benefits [19]. Viability of freeze dried probiotic cultures in different conditions has been well documented. However, there are only few reports [13, 20] available on the ability of freeze dried cultures to express their probiotic properties after long term storage. In the present study, Shigella inhibition assay was used to test the probiotic properties of freeze dried cultures during storage at 4 ^{0}C. Inhibition of Shigella growth was calculated by subtracting the Shigella viable cell count of tubes treated with freeze dried probiotic cultures from the viable cell count of the control tube with only Shigella as previously explained [21]. The result was expressed as $\log_{10}$ CFU/ml. According to the results, shown in table 2, microbes in all tested probiotic microbial mixtures were able to maintain their probiotic properties during the six month storage period tested. A gradual reduction of inhibition was observed with the increase of storage time for all five probiotic mixtures. However, this may not be due to loss of their probiotic properties during storage. That may be due to the different number of initial probiotic cells in the freeze dried microbial mixtures inoculated to Shigella cultures. These results are in agreement with some previous similar studies. In one of such study it has been reported the ability of different strains of Lactobacilli to maintain their probiotic properties during storage at 4 ^{0}C after freeze drying [20]. In another study, it has been reported the ability of a mixture of lactic acid bacteria and yeast isolated from Kefier to maintain their probiotic properties during storage at 4 ^{0}C after freeze drying [13].

Table 2: Difference in Shigella cell count of samples treated with different freeze dried microbial mixtures obtained at different days of storage at 4 ^{0}C and control Shigella cultures ($\log_{10}$ CFU/ml).

Microbial mixture	Days after freeze drying					
	0	30	60	90	120	180
in PBS	3.40 ± 0.09	3.34 ± 0.21	2.23 ± 0.14	1.89 ± 0.12	1.26 ± 0.08	1.38 ± 0.11
in UHT milk	6.59 ± 0.11	6.71 ± 0.08	5.48 ± 0.06	5.33 ± 0.04	5.12 ± 0.11	4.68 ± 0.09
in UHT milk + Sucrose	6.34 ± 0.16	6.04 ± 0.04	4.89 ± 0.23	5.22 ± 0.07	4.93 ± 0.05	4.24 ± 0.12
in UHT milk + Sorbitol	6.45 ± 0.07	6.23 ± 0.13	5.27 ± 0.11	5.05 ± 0.08	4.76 ± 0.20	4.33 ± 0.05
in UHT milk + Trehalose	6.20 ± 0.15	6.31 ± 0.06	5.06 ± 0.09	5.12 ± 0.12	4.84 ± 0.06	4.08 ± 0.14

4. CONCLUSION

This study contributes to the knowledge on the effect of freeze drying, long term storage and cryoprotectant used in freeze drying to the viability and probiotic properties of microbial mixtures. In conclusion, freeze drying of microbial mixtures in UHT milk is a good method for the preservation of those without reducing the cell viability and probiotic properties. Addition of other cryoprotectant to UHT milk is ineffective for the improvement of survival of tested probiotic organisms in freeze drying. Freeze drying or long term storage after freeze drying do not affect the probiotic properties of the tested microbes. These results will be useful in the production of commercial probiotic cultures and thereby it will aid to the development of new probiotic food products which are important in infectious disease prevention.

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