[BIO12] The influence of different protective agents on survival of *Lb. salivarius* i24 subjected to freeze-drying for production of live cell in powderized form

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Introduction

The use of probiotic microorganisms for food and pharmaceutical industries is mainly driven by the increasing consumer health awareness. Probiotic microorganism defined as "living microorganisms, which upon ingestion in certain number, exert health benefits beyond inherent basic nutrition" (Guarner *et al.*, 1998), play an important role in promoting and maintaining the host health (Salminen *et al.*, 1998). Therefore, considerable interest is being stimulated in the incorporation of the probiotic into functional foods and pharmaceutical products. Probiotic microorganisms selected for commercial use must retain their beneficial characteristics for which they were originally selected (Salminen *et al.*, 1998). Among the most important characteristics is the survival of these microorganisms during storage and rapid growth during manufacture. Hence, production and maintenance techniques must be established which maximize the storage stability, viability and activity of the bacterial cells (Cox *et al.*, 1978) during the preparation of the culture.

Although frozen the probiotic culture in liquid nitrogen exhibit maximal survival (Smittle *et al.*, 1974), but the expenses of this storage condition limits the use of this method. The most convenient and satisfactory method for the long-term preservation of cultures is lyophilization or freeze-drying under vacuum. This is a stabilizing process in which a solution of a substance is first frozen and then the quantity of the solvent is reduced, first by sublimation and then by desorption to a value that will no longer support biological activity or chemical reactions (Jennings and Duan, 1990). The result of lyophilization is greatly influenced by protective agent - single or mixture of substances, which protect the cells membrane against the effects of exposure to low temperature.

In this study, the effectiveness of different formulations of different protective agents such as skim milk, glycerol and sucrose as individual and a mixture to maintain viability of *Lb. salivarius* i24 during freeze-drying for production of live cell in powdered form was investigated.

Materials and methods

Microorganism

Lb. salivarius i24, isolated from chicken intestine (Jin *et al.*, 1996d), was used throughout this study. It was kindly provided by Digestive Microbiology Unit, Laboratory of Enzyme and Microbial Technology, Institute of Bioscience, Universiti Putra Malaysia.

Protective agent

The following compounds were tested for their protecting effect: 20% (w/v) skim milk, 20% (w/v) sucrose, 5% (v/v) glycerol, 17.80% (w/v) skim milk + 5.5% (w/v) sucrose, 16.55% (w/v) skim milk + 3.34% (v/v) glycerol + 9.01% (w/v) sucrose and distilled water as a control.

Preparation of samples

The bacterium was propagated statiscally in a bottle containing the growth medium (3.324% Glucose, 4.31% Yeast extract, 0.2% K_2HPO_4, 0.02% $MgSO_4$ and 0.1% Tween 80) and incubated at 37^{o}C. Cells in the early stage of stationary phase (15h were harvested under aseptic conditions by centrifugation for 5 min at 10,000 rpm (Eppendoof, centrifuge 5810R). Harvested cells were washed twice using 0.02M, pH 7.2 phosphates buffer. The cell pellets was then resuspended in the same volume of the desired protective agent. Each

suspension was transferred into a sterile universal bottle and chilled at 4°C for 2 h. Then, it was frozen for at least 18-20 h at –80°C prior to be freeze-dried for 24 h in a freeze-dryer (Labconco 195). Viable counts of cells were determined before freeze-drying and immediately after freeze-drying by pour plate method.

Results and Discussion

The suspension of *Lb. salivarius* i24 in distilled water without any protective agent before freeze-drying gave a very low survival rate (0.08%) immediately after freeze-drying (Table 1 and Fig 1. Surprisingly, similar result was also observed when the cell was suspended in glycerol as a protective agent. Even though glycerol is considered as one of the classical protective agent, but from the study it was found that the protective effect of glycerol to *Lb. salivarius* i24 cell was almost none. It scarcely exhibits a protective effect in this study. A reduction of 1.73 and 2.85-log cycle in viable cell count (Table 1) was observed after 1 month of storage at –80°C for the control and glycerol, respectively.

TABLE 1 The effect of various protective agents on the survival rate after freeze-dried and storage.

Protective agent	cfu/mL		% of survival	cfu/mL after 1 month	log cycle reduction
	Before FD	After FD			
Control (distilled water)	1.306×10^{10}	1×10^{7}	0.08	1.83×10^{5}	1.73
Skim milk	1.09×10^{10}	1.42×10^{9}	13.03	3.65×10^{8}	0.59
Sucrose	4.98×10^{9}	4.48×10^{8}	9.00	6.85×10^{7}	0.82
Glycerol	5.43×10^{9}	7×10^{2}	0.00	0	2.85
Sucrose + Skim Milk	1.306×10^{10}	5.11×10^{9}	39.10	2.10×10^{9}	0.38
Sucrose + glycerol + skim milk	1.306×10^{10}	7.21×10^{9}	55.17	2.93×10^{9}	0.39

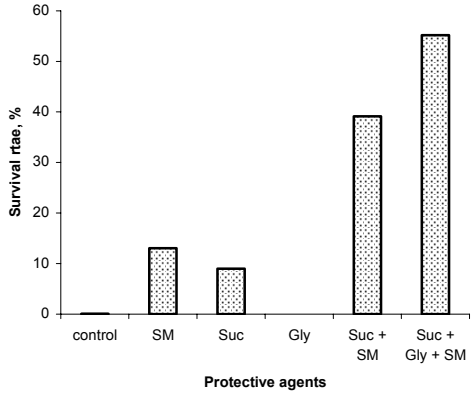

FIGURE 1 The survival rate of different protective agent after freeze-drying.

Results from this study also showed that cells freeze-dried with sucrose and skim milk had substantially higher survival rates compared with the control and the use of glycerol as a protective agent. Although sucrose showed a survival rate of 9% after freeze-drying, but the use of sucrose alone did not preserve the viability well during subsequent storage. Viable cell count declined by 0.82 log cycle after 1 month of storage. Skim milk offered 13.03% survival rate in freeze-drying and the number of viable cell count was decreased by only 0.59 log cycle during storage.

As expected, the two combinations of protective agents; (sucrose + skim milk) and (sucrose + glycerol + skim milk) gave a better protection when they were used together compare to individual. It was observed that the combination of sucrose, glycerol and skim milk gave better results in the freeze-drying (55.17%) than the other combinations (39.10%). Probably this result is due to intrinsic difference in the adding of glycerol. Since glycerol has already recognized as protective agent for lactic acid bacteria during freezing (Fonseca *et al.*, 2000). Even though in this study, glycerol showed no protective effect after freeze-drying, but it might be exhibited some protective effect during freezing step prior to freeze-drying when used together with other protective agents.

From Table 1, both combinations displayed a significant reduction in log cycle of 0.38 and 0.39 after 1-month storage. Skim milk solids are expected to prevent cellular injury by stabilizing cell membrane

constituents (Castrol *et al.*, 1995). In addition, milk proteins may form a protective coating on the cell wall proteins and calcium in milk, which increased the survival rate after freezing or freeze-drying (King and Su, 1993). It is desirable to prepare protective solutions that not only contain compatible solutes, but also buffering solutes for pH stabilization (Modler and Villa, 1993). Skim milk contains many solutes such as phosphates and citrate that would provide this buffering capacity. Mean while, sucrose and glycerol are good tonicity adjusters. Beside that sucrose also acting as a bulking agent and stabilizer. The purpose of bulking agent is to provide bulk to the formulation. On the other hand, it can also produce an elegant cake structure with good mechanical properties.

Conclusion

Lb. salivarius i24 was one of the important probiotic in the poultry industry. The best method for preserving this bacterium was through freeze-drying with suitable protective agents. Even though detrimental effects were recorded for all protective agents after freeze-drying. However, according to the result the survival improved when the protective agents were used as mixture instead of individual. The highest survival rate, 55.17% was obtained when the combination of sucrose, glycerol and skim milk was used.

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