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The Influence of Protective Agents in the Formulation of Freeze-Dried *Lactobacillus salivarius* I 24 on the Survival Rate During Storage

Lim Chi Ming[1], Murni Halim[1,2]*, Mohammad Rizal Kapri[2] and Arbakariya B. Ariff[2]

[1]Department of Bioprocess Technology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia

[2]Bioprocessing and Biomanufacturing Research Complex, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia

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Author : Murni Halim

Abstract

The exploitation of probiotic microorganisms particularly in the food industries is mainly owing to public acceptance and health awareness. The production of probiotic bacteria with a large number of uninjured and viable cells is important in ensuring their effective commercial applications. Liquid and frozen concentrates have been extensively used in the past, but the culture stability and savings in the costs of transportation and storage can be improved using freeze dried formulation. The effect of different protective agents on the viability of *Lactobacillus salivarius* I 24 subjected to freeze-drying and stored at different temperatures was studied. Several additives were tested as protective agents and the maximum viability after freeze-drying was obtained with the combination of skim milk and sucrose (65%). It was also found that of the temperature (-30, 4 and 30°C) tested, -30°C was the best temperature for the storage of freeze-dried *L. salivarius* I 24.

Keywords: Cryoprotectant; Freeze Drying; Lactic Acid Bacteria; Probiotic

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Correspondence:
Murni Halim, Department of Bioprocess Technology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia,
E-mail: murnihalim@upm.edu.my

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Introduction

Probiotics are generally defined as live microorganisms in which upon ingestion in an adequate number, confers health benefits on the host beyond inherent basic nutrition [1]. Probiotics play an important role in promoting and maintaining the host's health [2-3]. Nowadays, substantial interests are being developed in the incorporation of probiotic strains into numerous food, nutraceutical and pharmaceutical products. Nonetheless, to attain these beneficial effects, it is necessary to uphold a certain rate of cells viability throughout the processing, storage as well as consumption period. Therefore, production and maintenance procedures must be developed with the aim of maximizing the probiotic cells viability, stability, and activity [4].

Freeze drying or also known as lyophilization or cryodesiccation is one of the recognized methods for preserving microorganism and perishable material through dehydration process. In most cases the method has been proven to be excellent in terms of maintaining long-term viability and has made it easy and more convenient for storage and transport. Freeze-drying works by initially freezing the material followed by reducing the surrounding pressure and adding enough heat [5]. These steps will permit the frozen water in the material to directly sublime from solid to gas phase without going through the liquid state. In general, the viability of a freeze-dried strain can be retained from 5 to 35 years [6] if the cell concentration is maintained in between 106-1010 cell/mL before and after the freeze-drying process. Nevertheless, the survival rates usually differ according to genus and perhaps also diverge among species.

Biological systems have been found to be subjected to the action of several injuring factors upon cryogenic treatment [7]. The viability of microorganisms upon freezing and freeze-drying is also affected by the conditions of cultivation, the age of the culture, the species as well as the storage conditions [8-10]. To reduce the effects of unfavourable factors, the use of cryoprotectants such as sugars, amino acids, or natural biological substrate and polymers is recommended [11-15]. This is because, it has been recognized that the protective agents are the most important factor both in increasing the survival rate of the bacteria after freeze-drying and minimizing the death rate of the dried organisms during long storage [16]. The protective agents are considered to stabilize, in place of bound water, the cellular constituents located at or near the cell membrane.

In this present investigation, we studied the effect of using different protectants in the formulation of freeze-dried *Lactobacillus salivarius* I 24 on the survival rate during different storage temperatures.

Materials and Methods

Microorganism

Potentially probiotic strain, *L. salivarius* I 24, locally isolated from chicken intestine, was kindly provided by the Institute of Bioscience, Universiti Putra Malaysia [17]. A single colony of *L. salivarius* I 24 was picked from De-Man, Rogosa and Sharpe (MRS) agar plate and inoculated in a sterile MRS broth. The culture was incubated in an incubator shaker at 37°C, 250 rpm for 24 hours. The cells were then harvested by centrifugation at 12,857 x g, 4°C for 5 minutes. The supernatant was discarded whilst the cell pellet was resuspended in 10% (v/v) filter sterilized glycerol solution. The glycerol stocks were then kept at -80°C and used throughout the study.

Cultivation, harvesting and freeze-drying conditions

L. salivarius I 24 was grown in a 2 L stirred tank bioreactor (BIOSTAT B, B. Braun Biotech International, Germany) with a working volume of 1 L. Mixing in the bioreactor was facilitated by a 6-bladed turbine impeller installed with a rotary shaft placed at the center of the stainless-steel top-plate, agitated at 50 rpm. The culture was grown in a medium comprised of 33 g/L glucose, 43 g/L yeast extract, 0.2 g/L $MgSO_4$, 2 g/L K_2HPO_4, and 1 mL/L Tween-80.

After 15 hours of incubation (early stationary phase), cells were harvested under an aseptic condition by centrifugation (Centrifuge 5810R, Eppendorf, Germany) at 10,000 rpm for 5 minutes. Harvested cells were washed twice using 0.02 M phosphate buffer (*pH* 7.2). The supernatants were discarded and cell pellets were resuspended in an equal volume of the protective agent. A volume of 20 mL suspension was then aliquot into a sterile universal bottle and cooled at 4°C for 2 hours. Then, it was frozen at -80°C for at least 24 hours prior undergoing the freeze-drying process for 24 hours in a freezer-dryer (Labconco 195, Labconco, United States of America). A plating technique was used to determine the viable cells counts for samples before and after freeze-drying process.

Protective agents

The cryoprotectants tested in this study were skim milk, glycerol, sucrose, calcium carbonate (that was used as a source of Ca^{2+}) and a mixture of skim milk, sucrose and glycerol. Three different mixtures of protective agents at different concentration and ratio were prepared: a) 9.8% (w/v) skim milk and 10.6% (w/v) sucrose (SMSA); b) 17.8% (w/v) skim milk and 5.5% (w/v) sucrose (SMSB) and c) 16.5% (w/v) skim milk, 9.0% (w/v) sucrose and 3.3% (w/v) glycerol (SMSG).

Recovery of freeze-dried cells and storage study

Sterile MRS broth was used to reconstitute the freeze-dried *L. salivarius* I 24 to their original pre-freeze-dried volumes. The cultures were then incubated at 37°C for 30 minutes. The reconstituted samples were serially diluted and plated on MRS agar in triplicate. Plates were incubated in an incubator oven at 37°C for 48 h prior to determination of viable cells. The freeze-dried cells were then stored at -30, 4 and 30°C (considered as room temperature) for a period of 6 months. The viable cells were enumerated at time intervals. The percentage of cell survival rate (%) after freeze-drying process was quantified as follows:

Cells survival rate (%) = Total number of viable cells after freeze-drying/Total number of viable cells before freeze-drying*100

Analytical methods

Freeze-dried *L. salivarius* I 24 cells were reconstituted to their original pre-freeze-dried volumes by sterile MRS broth. The cultures were incubated at 37°C for 30 minutes for subsequent viability cell counts. Serial decimal dilutions of each sample in the range of 10-5 - 10-14 were prepared in sterile MRS medium and were then plated onto MRS agar plates. The plates were incubated in an incubator oven at 37°C for 48 hours. The colonies formed were counted and expressed as a Colony Forming Unit (CFU).

Results

The probiotic cells of *L. salivarius* I 24, which were freeze-dried with a mixture of skim milk and sucrose had significantly higher survival rate than the other mixture and individual protective agents as shown in Table 1. The mixture of 9.8% (w/v) skim milk and 10.6% (w/v) sucrose (SMSA) impart the highest degree of protection (65%) compared to the other mixture and individual protectants in which exhibited 51.1% with SMSB, followed by SMSG (27.1%), skim milk (13.0%) and sucrose (9.0%). Meanwhile, there was no protection against freeze-dried bacteria either using with glycerol or calcium carbonate.

The viabilities of freeze-dried *L. salivarius* I 24 stored at three different temperatures (-30, 4 and 30°C) for six months are shown in Table 2. Temperature of -30°C was the best condition for the storage of freeze-dried *L. salivarius* I 24, followed by 4°C and 30°C (considered as room temperature) was the worst, for all the tested protective agents (SMSA, SMSB and SMSG).

Discussion

Better preservation at -30°C can be ascribed to the lower reaction rates at this temperature compared to the other two temperatures (4°C and 30°C) studied. According to Bruno and Shah [18], as the storage temperature increases, mortality of the cultures also increases. In the present study, it was found that increases in the storage temperature resulted in an increase of the mortality of freeze-dried *L. salivarius* I 24 cultures. Previously, freeze-dried *L. acidophilus* LA-5® that were cryopreserved with skimmed milk demonstrated better cell survival when stored at 4°C than 25°C over a storage period of six months [19]. Similar results were reported by Savedboworn *et al.,* [20] who established a positive relationship between mortality and storage temperature of freeze-dried probiotic *Lactobacillus plantarum*. Using rice-protein-fructooligosaccharide as the protective agent, they observed the lowest specific rate of cell death for storage at 4°C compared to 30°C over a storage period of 180 days. Furthermore, the presence of protective agents effectively protects and enhances the cell functionality during exposure to gastrointestinal track conditions. The results of this study also confirmed the results of previous studies, which reported that temperature was a critical factor that influences the survival rate of microorganism during storage [21, 22]. Higher rates of survival were often obtained at low temperatures, especially at sub-zero cold storage.

The present study also showed that of the three different formulations of protective agents used, SMSA was better than SMSB or SMSG in maintaining the viability of freeze-dried *L. salivarius* I 24 during six months of storage. The highest protective effect was attained by SMSA with the survival rate of 65%. Although the compounds (skim milk and sucrose) in SMSA and SMSB were the same, their ratios were different. The ratio of skim milk to sucrose for SMSA was 0.92 (w/w) and 3.2 (w/w) for SMSB. This discloses that

Table 1: The viability and survival rates of freeze-dried *Lactobacillus salivarius* I 24 formulated with various protective agents.

Protective agent	Number of Viable cell (cfu/mL)		Percentage of cell survival (%)
	Before Freeze Drying	After Freeze Drying	
Distilled water (as control)	1.3×10^{10}	1.0×10^{7}	0.1
Skim Milk (20% (w/v))	1.1×10^{10}	1.4×10^{9}	13.0
Sucrose (20% (w/v))	5.0×10^{9}	4.5×10^{8}	9.0
Glycerol (5% (w/v))	5.43×10^{9}	7.0×10^{2}	1.3×10^{-5}
Calcium carbonate (0.5% (w/v))	4.1×10^{10}	0.00	0.0
SMSA (9.8 % (w/v) skim milk and 10.6 % (w/v) sucrose)	6.0×10^{10}	3.9×10^{10}	65.0
SMSB (17.8 % (w/v) skim milk and 5.5% (w/v) sucrose)	9.0×10^{10}	4.6×10^{10}	51.1
SMSG (16.5 % (w/v) skim milk, 9.0% (w/v) sucrose and 3.3% (v/v) glycerol)	2.1×10^{9}	5.7×10^{8}	27.1

Table 2: The effect of protective agents on the viability of *Lactobacillus salivarius* I 24 during storage at different temperatures for a six-month period.

Protective agent	Storage Temperature	Storage period (month)						
		0	1	2	3	4	5	6
SMSA	30°C	3.30×10^{9}	1.17×10^{8}	1.55×10^{7}	2.00×10^{7}	9.00×10^{6}	6.10×10^{5}	5.23×10^{4}
	4°C		1.38×10^{9}	1.05×10^{9}	3.50×10^{8}	5.55×10^{8}	1.01×10^{8}	6.12×10^{7}
	-30°C		2.33×10^{9}	2.37×10^{9}	7.40×10^{9}	7.78×10^{8}	6.62×10^{8}	5.10×10^{8}
SMSB	30°C	3.23×10^{9}	1.06×10^{5}	2.30×10^{2}	--	--	--	--
	4°C		1.05×10^{9}	1.38×10^{8}	3.50×10^{7}	1.01×10^{7}	5.72×10^{6}	3.02×10^{6}
	-30°C		2.33×10^{9}	2.37×10^{9}	7.40×10^{8}	4.12×10^{8}	7.10×10^{7}	9.12×10^{6}
SMSG	30°C	5.79×10^{9}	8.70×10^{4}	1.10×10^{2}	--	--	--	--
	4°C		5.20×10^{9}	3.69×10^{9}	1.89×10^{9}	6.02×10^{8}	4.10×10^{7}	3.10×10^{6}
	-30°C		5.89×10^{9}	3.45×10^{9}	2.57×10^{9}	9.78×10^{9}	5.17×10^{9}	4.01×10^{8}

SMSA (9.8% (w/v) skim milk and 10.6% (w/v) sucrose)
SMSB (17.8% (w/v) skim milk and 5.5% (w/v) sucrose)
SMSG (16.5% (w/v) skim milk, 9.0% (w/v) sucrose and 3.3% (v/v) glycerol)

the higher percentage of sucrose in the formulation imparts better protection. Two hypotheses have been postulated to elucidate the stabilizing effect of the disaccharides that are either based on water replacement or vitrification. In the water replacement hypothesis, cells stabilization is ascribed to the formulation of hydrogen bonds formed between sensitive components and disaccharide molecules whilst water is being removed [23, 24]. The structural integrity of the cell membrane and protein is thus maintained. Meanwhile, the vitrification hypothesis is correlated to the capacity of disaccharides to form a glassy structure when the material is drying under appropriate conditions [24] in which the sensitive components such as viable cells are embedded. The factor accountable for the long stability of biological materials is believed to be the glassy state of the matrix, characterized by extremely low molecular mobility. Hsi-Chia *et al.*, [25] suggested that sucrose protected the cells during freezing and drying phase since sucrose can inhibit free radical production in freeze-dried cultures. The deleterious effect of free radicals at the instant of rehydration is hence minimized. Furthermore, the protective effect of sucrose during freeze-drying process is also because it has low Gibbs free energy of solvation at low temperatures [26].

Conclusion

In can be concluded that among the individual cryoprotectants investigated, skim milk and sucrose are better than glycerol and Ca^{2+} in preserving *L. salivarius* I 24 cells from extreme conditions during freeze-drying process. The protective capacities could be further improved with a suitable formulation as a mixture component for instant by using higher percentage of sucrose than skim milk (i.e., 10.6% (w/w) sucrose and 9.8% (w/w) skim milk). -30°C was the best temperature for the storage of freeze-dried *L. salivarius* I 24, followed by 4°C and 30°C for all the protective agents (SMSA, SMSB, SMSG) tested.

Ethics Approval and Consent to Participate

Not applicable.

Competing Interests

The authors have declared no conflict of interest.

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