Direct RESEARCH JOURNALS

A comparative analysis of preservation of functional food cultures by freeze- drying, liquid-drying and freezing methods

Direct Research Journal of Agriculture and Food Science (DRJAFS) Vol.2 (2), pp. 13-18, February 2014
Available online at directresearchpublisher.org/drjafs
ISSN 2354-4147 ©2014 Direct Research Journals Publisher

Research Paper

Tan, G.H.[1]*and Mustapha, N.[2]

[1]Department of Agriculture Technology, Faculty of Agriculture, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia.
[2]Bioprocessing Program, Biotechnology Research Centre, Malaysian Agricultural Research and Development Institute, P.O. Box 12301, General Post Office 50774 Kuala Lumpur, Malaysia.

ABSTRACT

There are various methods for long term preservation of microorganisms, including freeze-drying (F-drying), liquid-drying (L-drying), and freezing at -80°C or at -196°C. All these methods have been developed and used to avoid degeneration and mutation of strains. L-drying involves vacuum drying of samples from the liquid state without freezing, and it is known to be useful for the preservation of microorganisms that are sensitive to freeze-drying. In this study, all types of functional food cultures were preserved by three methods: freeze-drying, liquid-drying and freezing at -20°C, -80°C and -196°C. The viability and stability of each culture was examined at different stages: before preservation, and six months after preservation storage. This study demonstrated that there were more than 80% of preserved cultures managed to grow after six months of storage in all methods. The success of long term preservation was always depends on the growth rate and desiccation tolerance of the microorganism itself. However, growth media and protective agent also play very important role in viability and stability of the cultures.

Key words: Freeze-drying,liquid-drying, freezing, viability, protective agent.

*Corresponding Author E-mail: geok hun@upm.edu.my; tangeokhun@gmail.com;
Tel: 603-89474893;Fax: 603-89381015

Accepted 11 January 2014

INTRODUCTION

A functional food is a food given an additional function (often one related to health-promotion or disease prevention) by adding new ingredients or more of existing ingredients. The general category of functional foods includes processed food or foods fortified with health-promoting additives, like "vitamin-enriched" products. Products considered functional generally do not include products where fortification has been done to meet government regulations and the change is not recorded on the label as a significant addition "invisible fortification". An example of this type of fortification would be the historic addition of iodine to table salt, or Vitamin D to milk, done to resolve public health problems such as rickets. Fermented foods with live cultures are considered functional foods with probiotic benefits. Functional foods have to be beneficially modulated target functions of the body, which are relevant to improve status of health or well-being, or reduction of disease risk.

Thus, collection and reliable preservation techniques of functional foods are important to ensure that the recovered cultures and other biological materials perform in the same way as the originally isolated culture. There are various methods for long term preservation of microorganisms, including freeze-drying (F-drying), liquid-drying (L-drying) and freezing at -80℃ and or -196℃ using liquid N_2. All these methods have been developed

Table 1. Solid media, cryoprotective agent (CPA) and rehydration fluid.

Media/ CPA/ rehydration fluid	Recipes, per litre
MRS	Casein peptone (10g), meat extract (10g), yeast extract (5g), glucose (20g), Tween-80 (1g), K_2HPO_4 (2g), Na-acetate (5g), $(NH_4)_2$Citrate (2g), $MgSO_4.7H_2O$ (0.2g), $MnSO_4.H_2O$ (0.05g), adjusted to pH 6.2-6.5
YMA	Yeast extract (10g), peptone (20g), dextrose (20g)
PDA	Potato (200g), corn sugar (20g), agar (20g)
Suspending media for bacteria (NBRC, NITE, Japan)	Sodium L (+)-glutamate monohydrate (3g), Ribitol (1.5g), L-cystein hydrochloride monohydrate (0.05g), 0.1M potassium phosphate buffer (100 ml, pH 7.0)
Suspending media for yeasts (NBRC, NITE, Japan)	Solution A:Polyvinylpyrrolidone (K-90) (6g), lactose (5g), distilled water (75 ml) Solution B: Sodium L (+)-glutamate monohydrate (3g), 1M potassium phosphate buffer (10 ml, pH 7.0), distilled water (15 ml) Sterilized separately by autoclaving, then mix solution A and B
Suspending media for filamentous fungi (NBRC, NITE, Japan)	Sodium L (+)-glutamate monohydrate (3g), 0.1M potassium phosphate buffer (100 ml, pH 7.0)
Rehydration fluid for bacteria (NBRC, NITE, Japan)	Polypeptone (10g), yeast extract (2g), $MgSO_4.7H_2O$ (1g), distilled water (1L)
Rehydration fluid for yeasts (NBRC, NITE, Japan)	Glucose (10g), peptone (5g), yeast extract (3g), malt extract (g), distilled water (1L), adjusted to pH 6.0
Rehydration fluid for filamentous fungi (NBRC, NITE, Japan)	Peptone (5g), yeast extract (3g), $MgSO_4.7H_2O$ (1g), distilled water (1L), adjusted to pH 6.0

and utilized to avoid degeneration and mutation of cultured desired microorganisms. In fact, freeze-drying or known as lyophilization is a dehydration process used to preserve microbial cells by freezing and reducing the surrounding pressure to allow the frozen water in the cells to sublimate directly from the solid state to the gas state (Day and Stacey, 2007). On the other hand, liquid-drying involves vacuum-drying of sample from the liquid state without freezing. This method was useful for the preservation of microorganisms which are sensitive to freeze-drying (Anner, 1970; Imai and Sakane, 1985; Malik, 1990; Sakaneet al., 1992). Likewise, cryopreservation (freezing at -80°C or -196°C) is a technique by using very low temperature to preserve structurally intact living cells and tissues through cryoprotective agent (CPA), including dimethyl-sulfoxide (DMSO), mono-hydric alcohol and its derivatives [methanol (CH_3OH), ethanol (C_2H_5OH), polyvinyl alcohol(PVOH)], diols and derivatives [propylene glycol ($C_3H_8O_2$), polyethylene glycol (PEG), triols (glycerol, mannitols), monosaccharides (glucose, xylose), etc (Hubalek, 2003).

Besides the above, long term preservation methods have been improved and applied to preserve various types of bacteria, yeast and filamentous fungi and are mainly used in various foods and agriculture. The purpose of the present study was to examine the improved preservation techniques on microbial culture in Collection of Functional Food Culture (CFFC), Malaysian Agricultural Research and Development Institute (MARDI), Malaysia.

MATERIALS AND METHODS

Microorganisms and growth conditions

All microorganisms (bacteria, yeast and filamentous fungi) that were grown on appropriate media are shown in Table 1.

Preparation of protective agent for cryopreservation

Solution of the most effective protective agents, like glycerol (15%; v/v, Sigma Aldrich), was prepared in distilled water, filtered, sterilized and stored at 4 °C. The solution was closed tightly and autoclaved at 121 °C for 15 min.

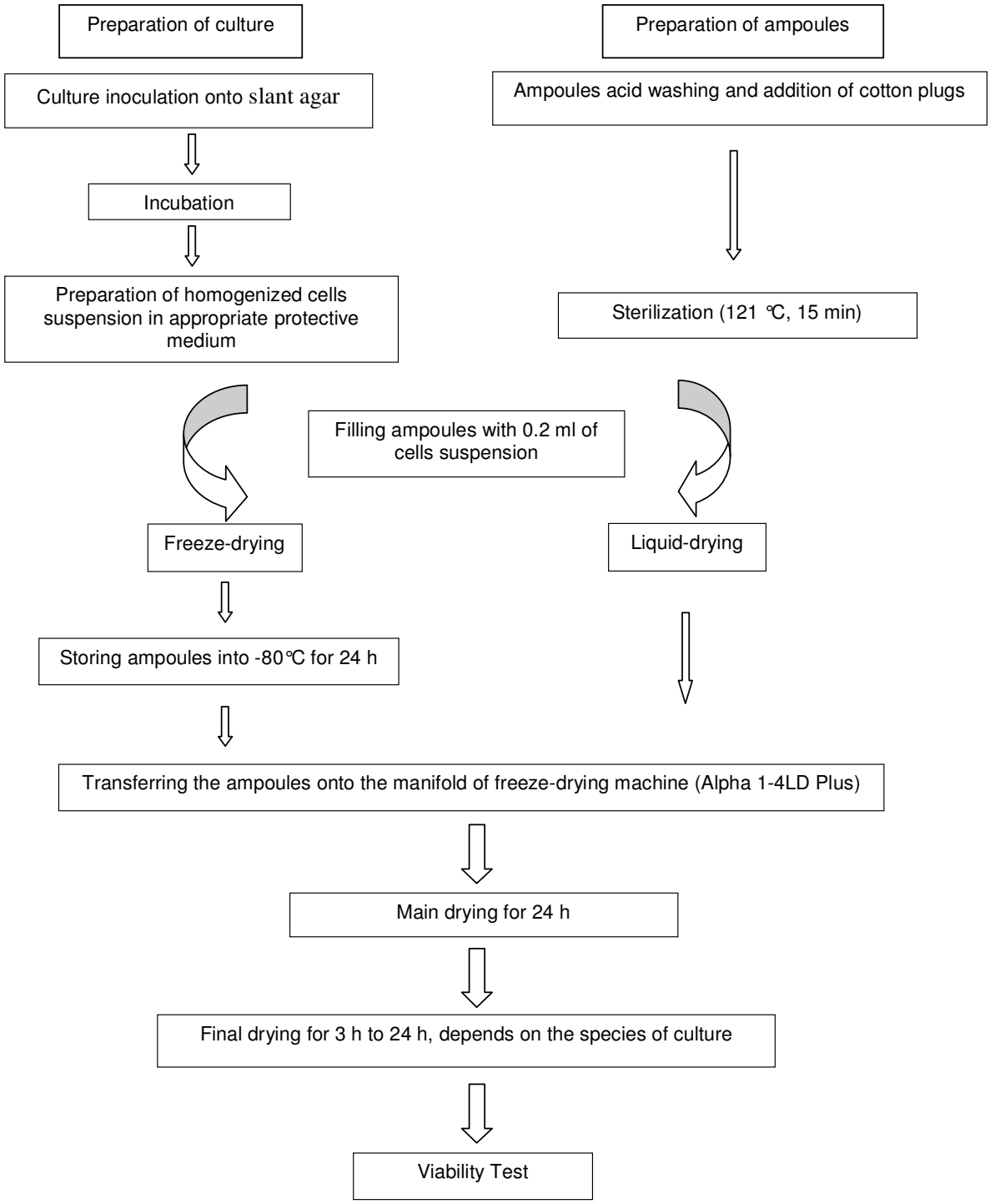

Figure 1: Flow diagram of freeze-drying and liquid drying process.

Preparation of cells suspension for cryopreservation

The bacteria, yeast and filamentous fungi were grew on slant agar. Cryoprotective medium (4 ml) was added to the culture slants using Pasteur pipette and mixed gently to make a suspension. 1 ml of suspension was transferred to each of the labeled cryo tube. The cryo tubes were placed into the boxes and stored in freezer to

maintain the temperature before transferred to -20°C, -80 °C and later to -196 °C (liquid N containers, Nalgene, USA).

Preparation of protective agent for freeze-dying and liquid-drying

Solutions of the most effective protective agents, like non -fat milk solid (5%; w/v), sodium glutamate monohydrate ($C_5H_8NNaO_4.H_2O$) (5%; w/v, Sigma Aldrich) and Polyvinylpyrrolidone (K-90; 5%, w/v, Sigma Aldrich) were prepared in distilled water, filtered, sterilized and stored at 4 °C. The solution was boiled and the bottle was closed tightly and autoclaved at 121 °C for 15 min.

Preparation of cell suspension for freeze-drying and liquid-drying

The bacteria, yeast and filamentous fungi were grown on slants agar. Suspending medium (4 ml, Table 1 for composition) was added to the culture slants using Pasteur pipette and mixed gently to make a suspension. 0.2 ml of suspension was transferred to each of the labeled ampoule.

The top section of cotton plug was cut off with sterilized scissors, and pushed down about 4 cm down the ampoule. For liquid-drying, the ampoule was constricted about 1 cm above the cotton plug to produce a short and curved capillary section of about 2 cm in diameter. However, the samples for freeze-drying were kept at -80 °C for 24 hours.

Freeze-drying and liquid-drying procedure

The outline of the freeze-drying and liquid-drying procedure and the major steps involved are shown in Figure 1. The refrigerator of freeze-drying machine (Alpha 1-4LD Plus Freeze-dryer, Martin Christ, Germany) was switched on and the condenser drain valve was closed. When the condenser temperature was below -50°C, the main drying will take place. Main drying was carried out constant weight basis for 24 hours and continues until final drying. The ampoules were sealed after the freeze-drying was completed.

Revival of cultures from frozen cultures, freeze-dried and liquid-dried ampoules

The contents of the cryo tubes, freeze-dried and liquid-dried ampoules were recovered, reconstituted with sterile liquid growth medium (Table 1) and added into suitable microbial growth medium. Freshly inoculated cultures were incubated at 35 °C for 48 hours.

Estimation of viability and stability

The survival recoveries were checked before preservation, immediately after preservation and after six months of storage. For comparison, the viable cell counts before preservation, after preservation and during storage were calculated and percentage survival was determined following appropriate technique (Malik, 1990).

RESULTS AND DISCUSSION

A total of 119 microbial cultures were preserved for long term storage in Collection of Functional Food Culture (CFFC). Most of these cultures were potential as starter culture for food fermentation, such as*Rhizopusoligosporus* for tempe production, *Aspergillusoryzae* for soy sauce, *Acetobacterxylinum* for nata de coco, *Amylomycesrouxii* for tapai, several lactic acid bacteria (*Lactobacillus plantarum*, *Streptococcus thermophilus* and *Lactobacillus fermentum*) or yoghurt production.

Based on three different techniques of preservation, the viability and stability of microbial cultures were not significantly different. More than 80 % of preserved cultures managed to grow after six months of storage time (Table 2). The efficiency of cryopreservation in -196 °C is better than those of storage in -80 °C. From the study, the microbial cultures were stored slowly into -196 °C (liquid N tank). The loss of viability of certain microbes when stored at -20 °C may have been due to the damages to the cells. Based on the study from Grover et al., 1967, at the storage range of -10 to -40 °C, most of the cells contents may not have been frozen fully, and the concentration of electrolytes may become very high in the unfrozen parts (Bridge, 1966).

In the preservation study, there are many factors which can affect the success of long term preservation, viability and stability of microbial cultures. Growth rate of microorganisms itself could be the main factor. The optimal growth phase for desiccation survival has been found to be largely dependent on the organism (Morgonet al., 2006). For example, the stationary phase cells of *Lactobacillus rhamnosus* appeared to have given the highest recovery after having been dried, of 31–50%. Nonetheless, early log phase cells showed 14% survival and lag phase cells showed the highest susceptibility, with only a 2% cell survival (Corcoran et al., 2004). Similar results have been reported by Mary et al. (1986), where significantly higher cell viability was obtained from stationary phase Rhizobia cells thanthe corresponding exponential phase cells. In contrast, higher survival rates of *Sinorhizobium* and *Bradyrhizobium* were demonstrated when sampled in the lag phase of growth (Boumahdiet al., 1999).

Growth base and protective agent may be another factor which affects the viability and stability of microbial

Table 2.Survival of various microorganisms after preservation and storage period.

Genus	No of tested strains	Viability rate (%)[a]					
		Before	6 months storage at -20°C	6 months storage at -80°C	6 months storage at -196°C	6 months F-drying storage	6 months L-drying storage
(a) Bacteria							
i. Acetobactersp	7	100	86	90	95	95	98
ii. Acetobacterxylinum	2	100	69	70	80	85	90
iii.Lactobacillus acidophilus	6	100	98	98	100	100	100
iv. Lactobacillus brevis							
v.Lactobacillus plantarum	4	100	99	99	100	100	100
vi. Streptococcus sp	20	100	95	95	100	100	100
vii. Pediococcussp							
	7	100	95	96	100	100	100
	19	100	95	97	100	100	100
(b) Yeast							
i. Saccharomyces sp	17	100	98	99	100	100	100
ii.Schizosaccharomycessp	2	100	99	99	100	100	100
iii.Saccharomycopsissp							
iv. Candida sp	5	100	97	97	100	100	100
v. Hansenulasp							
vi.Zygosaccharomycesrouxii	4	100	90	90	99	100	100
vii.Torulopsisversatilis	3	100	99	99	100	100	100
	1	100	95	97	100	100	100
	1	100	90	90	90	90	100
(c) Fungi							
i. *Rhizopusoligosporus*	4	100	100	100	100	100	100
ii. *Amylomycesrouxii*	6	100	100	100	100	100	100
iii. *Aspergillusoryzae*	3	100	100	100	100	100	100
iv.*Monascuspurpureus*	8	100	100	100	100	100	100
Total	119						

[a] Percentage indicate average colony forming unit.

cells. Two recent studies have shown that the growth media can have a significant effect on the freeze-dried survival of *Enterococcus faecalis*, *Enterococcus durans* and *Lactobacillus bulgaricus* (Carvalhoet al., 2003, 2004). *Lactobacillus bulgaricus* showed the lowest decrease in viability after having been freeze-dried when it was grown in the presence of mannose, compared to those of fructose, lactose and or glucose. Antiseptic agents can be added during growth of the microorganisms, or prior to freezing or drying.

The level of cell viability after preservation was found to be based on the efficacy of the protective agent(s) used during the process.Morgonet al., (2006) have shown that during microbial growth, they can induce tolerance to certain adverse condition. For example, when the pH of the culture medium of *Lactobacillus reuteri* was reduced to 5, it induced a greater protection to the cells during freeze-drying, and producing a 90% cell recovery compared to those of 65%, when the cells were grown at pH 6. A similar response was found by Maus and Ingham (2003), where acid and cold tolerance was induced in probiotic strains of *Bifidobacterium*, by starving the cells in growth media kept at 6 ℃ or reducing the pH of the growth medium from 6.2 to 5.2 (Maus and Ingham, 2003).

Conclusion

Long term preservation of microbes always depends on the growth rate and desiccation tolerance of the microorganism itself. However, growth media and protective agent also play very important role in viability and stability of the cultures.

ACKNOWLEDGEMENTS

The authors would like to thank Malaysian Agricultural Research and Development Institute, Selangor, Malaysia for RMK10 Grant. Many thanks to Dr.Kamariah Long and all CFFC members for fully support in this project.

REFERENCES

Annear DI (1970). A note on the preservation of soil amoebas by drying *in vacuo*. *Cryobiology* 7:129-131.

Boumahdi M, Mary P, Hornez JP (1999). Influence of growth phases and desiccation on the degrees of unsaturation of fatty acids and the survival rates of rhizobia.*Journal of Applied Microbiology* 87: 611–619.

Bridges BA (1966). Preservation of microorganisms at low temperatures. *Lab. Pract*. 15:418-422.

Carvalho AS, Silva J, Ho P, Teixeira P, Malcata FX, Gibbs P (2003). Effect of various growth media upon survival during storage of freeze-dried Enterococcus faecalis and Enterococcus durans. *Journal of Applied Microbiology* 94:947–952.

Carvalho AS, Silva J, Ho P, Teixeira P, Malcata FX, Gibbs P (2004). Effects of various sugars added to growth and drying media upon thermo tolerance and survival throughout storage of freeze dried *Lactobacillus delbrueckii ssp. bulgaricus*. *Biotechnology Progress.* 20:248–254.

Corcoran BM, Ross RP, Fitzgerald GF, Stanton C (2004). Comparative survival of probiotic lactobacilli spray-dried in the presence of prebiotic substances. *J.Appl. Microbiol*. 96:1024–1039.

Day JG, Stacey GN (2007). Cryopreservation and freeze drying protocol.*Humana Press*.365 pp.

Grover AA, Kim HK, Wiegeshaus EH, Smith DW (1967). Host-parasite relationships in experimental airborne tuberculosis II. Reproducible infection by means of an inoculums preserved at -70°C. *Journal of Bacteriology* 94(4):832-835.

Hubalek Z (2003). Protectants used in the cryopreservation of microorganisms.*Cryobiology* 46:205-229.

Imai K Sakane T (1985). Preservation of chemolithotrophic bacteria by L-drying.*Fundamentals and applications of freeze-drying to biological materials, drug and foods stuffs*, pp. 233-234, Int. Inst. Refrig., Commission C1, Paris, France.

Malik KAA (1990).A simple liquid-drying methods for the preservation of microorganisms sensitive to freezing and freeze-drying.*Journal of microbiological methods* 12:125-132.

Mary P, Ochin D, Tailliez R (1986).Growth status of rhizobia in relation to their tolerance to low water activities and desiccation stresses. *Soil Biology and Biochemistry* 18(2):179–184.

Maus JE, Ingham SC (2003). Employment of stressful conditions during culture production to enhance subsequent cold-and acidtolerance of bifidobacteria. *Journal of Applied Microbiology* 95:146–154.

Morgon CA, Herman N, White PA, Vesay G (2006). Preservation of microorganisms by drying: A review. J.Microbiol. Methods 66:183-193.

Sakane T, Fukuda I, Itoh T, Yokota I (1992). A. Long term preservation of halophilicarchaebacteria and thermoacidophilicarchaebacteria by liquid drying.*J.Microbiol.Methods* 16:281-287.