FREEZE-DRYING OF STARTER CULTURES

By W. F. SCHUBERT, B.A., B.Sc., Dip. Ed., Biochemist,

Research Branch, Division of Dairying.

SUMMARY.

Cultures dried by the Rayner method retained their vitality for up to seven months, but in most cases a high cell mortality accompanied the drying.

Self-frozen cultures prepared with Gray's apparatus gave lower survival populations than externally frozen cultures. The mortality on storage is high in all drying procedures.

I. INTRODUCTION.

Many biological fluids, including suspensions of bacteria, contain heat-labile proteins and other substances which lose their biological activity when highly concentrated or subjected to temperatures in excess of 30 deg. C. Bacterial suspensions cannot therefore be preserved by drying from aqueous solutions.

As early as 1909 Shackell suggested that deterioration of labile material at high concentrations and elevated temperatures could be overcome if the solutions were dried from the frozen state and then placed over a desiccant in a high vacuum. Under these circumstances evaporation was sufficiently rapid for the solution to remain frozen. Drying then occurred by ice sublimation from the frozen state without an intermediate liquid phase. This process of freezing and drying provides the only reliable method of preserving labile materials such as bacteria. The method has been widely used both in the laboratory and in industry for the preservation of cheese starter organisms.

The purpose of the present investigation was to examine some of the methods available in a small laboratory for the freezing and drying of "starter" cultures and to examine some of the factors upon which the efficiency of freezing and drying depends.

II. GENERAL CONSIDERATIONS.

(1) Fundamental Requisites.

Flosdorf, Hull, and Mudd (1938) list the requisites of the technical set-up for freeze-drying as follows:—

(*a*) A device to ensure the maximum rate of evaporation from the material undergoing desiccation which is compatible with the total operation and with the safety of the product.

(*b*) A device which ensures the maximal rate of flow of water vapour from the evaporating surface to the surface where the vapour is removed.

(*c*) A device to achieve the maximum economy and efficiency of removal of water vapour whether by condensation or evaporation.

These requisites may be satisfied in a variety of ways and consequently considerable differences in technical design are in evidence in the methods employed for the freeze-drying of bacterial cultures.

(2) Freezing of Culture.

The first step in the freeze-drying process is the freezing of the culture to be dried. This may be achieved either by self-freezing or by the application of an external freezing mixture. When self-freezing is practised, preliminary degassing must be employed to avoid frothing. This may be accomplished by shutting off the vacuum pump for a minute or two as soon as the fluid in the ampoules commences to bubble. When external freezing is employed, a temperature below the eutectic must be used; otherwise the liquid phase which separates from the frozen remainder will not only froth violently at the subsequent evacuation stage but the culture will be liquid-dried rather than freeze-dried, with consequent loss of biological activity (Bradish 1947).

(3) Vacuum Sublimation of Ice.

In any sublimation process a solid substance is vaporized in such a way that the intermediate liquid phase is eliminated. The vapour which is removed is disposed of in some other part of the apparatus, while the non-subliming substances are retained in the ampoules. Vacuum sublimation is employed since the elimination of the liquid phase is a critical factor. The water vapour may be disposed of by being condensed at a low temperature, by the use of desiccating substances such as P_2O_5, H_2SO_4 and/or silica gel, or by direct pumping.

The efficiency with which vacuum sublimation can be carried out is a function of many physical variables.

(4) Design of Equipment.

In the range 10 to 1,000 microns, where drying by sublimation occurs, resistance to flow is inversely proportional to the fourth power of the radius and directly proportional to the length of the line. Thus a piece of apparatus should be so designed that there are few or no restrictions to the flow. This is not easy to accomplish in practice and some of the apparatus used in the experimental work described in this paper suffers in some respects from this deficiency.

III. FREEZE-DRYING OF "STARTER" CULTURES.

The freeze-drying of bacterial cultures is not merely of academic significance. It has found many applications in the fermentation industries,

including the dairying industry. Traditionally, "starters" used for the manufacture of cheese have been distributed to factories in chalk-litmus-milk. This method has proved very satisfactory and is widely used in many localities. But in recent years dairy laboratories have adopted the practice of preserving stock cultures in the dried state and of distributing cultures to cheese factories in the dried or powdered form. The Dairy Research Section of the C.S.I.R.O. commenced an Australia-wide distribution of cultures in this form in January, 1955.

IV. OUTLINE OF EXPERIMENTAL WORK.

(1) Using the Rayner Method.

Using the Rayner method the following investigations were made:—

(*a*) The effect of storage on vitality and morphology.

(*b*) The determination of survival populations after 5, 6 and 7 months' storage respectively.

(2) Using Gray's Method.

Using Gray's method the following investigations were made:—

(1) The determination of the effect of freezing and drying on survival populations using self-freezing.

(2) The determination of the effect of freezing and drying on survival populations using external freezing.

(3) The determination of strain susceptibility to the effects of freezing and drying.

(4) The effect of pre-centrifuging of cultures on survival populations.

V. EXPERIMENT USING RAYNER'S METHOD.

(1) Rayner's Method.

The cultures to be dried are grown overnight in sterile skim-milk. Drops of the culture are transferred by means of a capilliary pipette to sterile pieces of Cellophane (approx. $\frac{3}{8}$ in. square) resting in a sterile petri dish.

The dish and its contents are then transferred to a vacuum desiccator containing silica gel, the lid of the dish being left slightly open. The vacuum desiccator is then exhausted by means of a pump. The cultures freeze and dry within 20 minutes. The cultures are nevertheless left overnight in the exhausted desiccator. The Cellophane strips are then transferred to sterile ampoules; these are placed in the desiccator, which is again evacuated and left overnight. The ampoules are then evacuated and sealed at the constriction in a blow-pipe flame.

After sealing, each tube of dried culture was tested for vacuum (to ensure that there were no leaks) by means of a high frequency vacuum tester

connected to the A.C. mains supply. Any ampoules exhibiting crazing were discarded, as it was found that such ampoules did not hold their vacuum very well.

(2) Effect of Storage on Vitality and Morphology.

(*a*) *Preparation of cultures.*—The cultures used were:—"GB", "H.P.", "Q_4", "R_1", "S_1", "ML_2", "ML_3", "F_1", and "MHS_4". The cultures were inoculated into sterile skim-milk and incubated for 24 hr. at 22 deg. C. preparatory to being dried.

Vitality test:—The cultures were subjected to the following vitality test. Milk pasteurised at 145 deg. C. for 30 min. is dispensed in 150 ml. quantities and the temperature raised to 30 deg. C. Then 1 per cent. starter is added and the starting time of the test is calculated from this point. Thirty minutes later five drops of rennet are added and one hour later the curd is cut to ¼ in. cubes with a knife. The temperature is then raised to 37 deg. C., which is maintained for two hours. The whey is then drained from the curd, and incubation at 30 deg. C. for a further hour follows. Nine millilitres of whey are titrated with ·1N NaOH, using phenolphthalein as an indicator.

(*b*) *Microscopic examinations.*—These were conducted by inoculating tubes of sterile skim-milk with one loopful of culture, incubating at 22 deg. C. and 37 deg. C. respectively. After four hours' incubation smears are prepared, stained with Newman's stain and examined microscopically.

Vitality tests and microscopic examinations were conducted on cultures before and after storage for periods up to seven months. No fewer than eight cultures were dried in each trial. Results and observations are recorded in Table 1.

In cases where clotting did not occur, the ampoules had not held their vacuum or the cultures had very poor initial vitality. The vitality of the "starters" did not appear to be appreciably affected by drying and storage in the dried state for periods up to seven months.

With one or two exceptions, morphology did not appear to be affected by drying and storage.

(3) Determination of Survival Populations after Storage.

The determination of viable counts before and immediately after freeze-drying and after storage, though subject to certain inaccuracies, does afford some information concerning the effect of drying on the initial population. Viable counts before and after storage for five, six and seven months are tabulated in Table 2. Though all cultures exhibited a high mortality on drying and storage, clotting nevertheless occurred in all cases within 36 hr. The viable counts were made on Hunter's media (1946), which gave large, easily countable colonies of the "starter" organisms.

Table 1.

RELATIONSHIP BETWEEN STORAGE TIME, VITALITY AND MORPHOLOGY.

Storage Period (months).	Culture.	Vitality Test.		Morphology.			
		Before Freeze-drying.	After Freeze-drying.	Before Freeze-drying.		After Freeze-drying.	
				At 22°C.	At 37°C.	At 22°C.	At 37°C.
3	GB	26	27	Typical	Typical	Typical	Long chains
	HP	35	35	,,	,,	,,	Typical
	Q4	25	40	,,	,,	,,	,,
	R1	19	29	,,	,,	,,	,,
	S1	3	..	,,	,,	,,	,,
	ML2	19	20	,,	,,	,,	,,
	ML3	29	31	,,	,,	,,	Long chains
	MHS4	27	28	,,	,,	,,	Typical
4	GB	30	35	Typical	Typical	Typical	Typical
	HP	30	46	,,	,,	,,	,,
	Q4	32	24	,,	,,	,,	,,
	R1	21	30	,,	,,	,,	,,
	S1	3	3	,,	,,	,,	,,
	ML2	4	2	,,	,,	,,	,,
	ML3	14	15	,,	,,	,,	,,
	MHS4	44	..	,,	,,	,,	,,
5	GB	36	27	Typical	Typical	Typical	Typical
	HP	39	34	,,	,,	,,	,,
	Q4	8	..	,,	,,	,,	,,
	R1	33	20	,,	,,	,,	,,
	S1	7	3	,,	,,	,,	,,
	S2	24	..	,,	,,	,,	,,
	ML2	31	24	,,	,,	,,	,,
	ML3	33	39	,,	,,	,,	,,
	F1	12	8	,,	,,	,,	,,
6	GB	32	..	Typical	Typical	Not examined as cultures possessed no vitality	
	HP	36	..	,,	,,		
	Q4	42	..	,,	,,		
	R1	25	..	,,	,,		
	S1	12	..	,,	,,		
	S2	18	..	,,	,,		
	ML2	14	..	,,	,,		
	ML3	33	..	,,	,,		
7	GB	32	29	Typical	Typical	Typical	Typical
	HP	31	39	,,	,,	,,	,,
	Q4	4	40	,,	,,	,,	,,
	R1	30	37	,,	,,	,,	,,
	S1	1	15	,,	,,	,,	,,
	S2	14	4	,,	,,	,,	,,
	ML2	22	17	,,	,,	,,	,,
	ML3	39	39	,,	,,	,,	,,

Table 2.

SURVIVAL POPULATIONS AFTER 5, 6 AND 7 MONTHS.

Storage Period. (months).	Culture.	Bacterial Estimates.	
		Before Freeze-drying.	After Freeze-drying.
5	GB	89,000,000	80,000
	HP	36,500,000	10,000
	Q4	28,400,000	10,000
	R1	49,200,000	10,000
	S2	8,500,000	10,000
	S3	L.A.	10,000
	ML2	47,000,000	40,000
	ML3	52,000,000	70,000
	F1	24,000,000	10,000
6	GB	48,000,000	10,000
	HP	19,000,000	10,000
	Q4	17,200,000	10,000
	R1	30,700,000	10,000
	S1	4,200,000	10,000
	S2	19,300,000	10,000
	ML2	30,900,000	10,000
	ML3	44,800,000	10,000
7	GB	52,700,000	970,000
	HP	32,600,000	10,000
	Q4	18,800,000	10,000
	R1	27,400,000	10,000
	S1	18,300,000	70,000
	S2	20,900,000	40,000
	ML2	36,000,000	100,000
	ML3	41,000,000	530,000

(4) Conclusions.

(*a*) The Rayner method requires only simple equipment and does not involve complex techniques.

(*b*) Cultures dried by this method retained their vitality for periods up to seven months.

(*c*) Drying was accompanied by a high cell mortality in practically all cases.

VI. EXPERIMENTS USING GRAY'S METHOD.

(1) Gray's Method.

Gray's apparatus consists of a hollow metal cylinder 18 in. high and 4 in. in diameter. The cylinder is sealed at the base and is fitted with a detachable vacuum-tight lid, similar in construction to the type commonly fitted to the McIntosh and Fildes anaerobic jar.

Several rows of metal ports communicating with the hollow centre and sloping slightly downward from the horizontal are arranged peripherally round the cylinder. A short length of pressure-tubing fitted with a release-type screw clamp is attached to each part.

The core of the cylinder is fitted with a nickelled copper gauze basket charged with silica gel. The moisture-laden air is drawn from the drying cultures by a central manifold. This air passes directly through the silica-gel in the basket. The exhaust manifold is fitted by means of a sleeve valve at the base of the cylinder to the exhaust pump.

Ampoules containing the cultures to be dried are inserted into the rubber parts, using a smear of lubricant, and the clips are loosened. Should the cultures froth during evacuation the pump is shut off for a minute or two.

(2) Determination of the Effect of Freezing and Drying on Survival Populations using Self-freezing.

Cultures were prepared as in previous experiments. The cultures were not pre-frozen in an external freezing mixture prior to the pump being switched on. Viable counts were made on the cultures prior to freeze-drying and then immediately after drying. The results obtained are shown in Table 3.

Survival rates on the whole were poor and rather erratic. A possible explanation of this is that some of the cultures were dried from the liquid and not from the frozen state.

Table 3.

PERCENTAGE SURVIVAL (AFTER DRYING—NO PRE-FREEZING).

Trial No.	Culture.	Viable Count, per 0·1 ml.		Percentage Survival.
		Before Freeze-drying.	After Freeze-drying.	
1	GB	475,000,000	289,000,000	61
	HP	789,000,000	38,000,000	5
	Q4	263,000,000	81,000,000	31
	R1	791,000,000	230,000,000	29
2	GB	1,000,000,000	1,047,000,000	100
	HP	1,222,000,000	332,000,000	27
	Q4	890,000,000	114,000,000	13
	R1	780,000,000	421,000,000	54
3	GB	2,080,000	1,200,000,000	58
	HP	1,160,000	134,000,000	12
	Q4	1,060,000	148,000,000	14
	R1	10,000,000	1,000,000	10
4	GB	1,960,000	701,000,000	36
	HP	1,210,000,000	1,000,000	1
	Q4	720,000,000	135,000,000	19
	R1	1,410,000,000	284,000,000	20

(3) Determination of the Effect of Freezing and Drying on Survival Populations using External Freezing.

The results of this experiment are recorded in Table 4. It will be observed that the figures are of a higher order and more uniform than those recorded in Table 3. Proper external freezing seems to ensure that the drying takes place from the solid and not from the liquid state, thereby ensuring that the biological activity of the system is preserved to a greater extent. The freezing mixture consisted of alcohol and dry ice.

Table 4.

PERCENTAGE SURVIVAL AFTER DRYING (WITH PRE-FREEZING).

Trial No.	Culture.	Viable Count per 0·1 ml.		Percentage Survival.
		Before Freeze-drying.	After Freeze-drying.	
1	GB	2,190,000,000	784,000,000	36
	HP	1,130,000,000	350,000,000	31
	Q4	440,000,000	92,000,000	21
	R1	1,030,000,000	259,000,000	25
2	GB	2,150,000,000	738,000,000	34
	HP	400,000,000	140,000,000	35
	Q4	520,000,000	104,000,000	20
	Q1	990,000,000	380,000,000	38

(4) Determination of the Influence of Pre-freezing on the Survival Populations of two Strains.

Two cultures—"GB", a lactis strain, and "HP", a cremoris strain—were pre-frozen by external freezing and dried. Counts were made before drying and immediately after drying to determine if any strain susceptibility to drying existed. The techniques employed were as described previously. Results are tabulated in Table 5.

On the whole, survival rates were high. "GB" exhibited more favourable results than "HP". However, this may be related more to morphology than to strain sensitivity to the effects of freezing and drying.

(5) Drying of Centrifuged and Uncentrifuged Cultures.

The centrifuged culture was prepared as follows. On the first day the culture was sown in 10 ml. sterile skim-milk and incubated at 22 deg. C. for 24 hours. On the second day, 1 ml. of the clotted culture was sown in 5 ml. of Hunter's broth and incubated. On the fourth day the broth culture was well shaken, transferred to a 15 ml. sterile centrifuge tube, and centrifuged at 3,000 r.p.m. for 30 min.

Table 5.

SURVIVAL POPULATIONS OF "GB" AND "HP".

Trial No.	Culture.	Viable Count per 0·1 ml.		Percentage Survival.
		Before Freeze-drying.	After Freeze-drying.	
1	GB	420,000,000	205,000,000	49
		390,000,000	219,000,000	56
		460,000,000	247,000,000	54
		380,000,000	254,000,000	67
2	HP	120,000,000	35,000,000	31
		160,000,000	32,000,000	50
		90,000,000	42,000,000	47
		180,000,000	34,000,000	19
3	GB	330,000,000	193,000,000	58
		300,000,000	225,000,000	75
		310,000,000	194,000,000	63
		330,000,000	152,000,000	46
4	HP	100,000,000	46,000,000	46
		100,000,000	59,000,000	59
		120,000,000	25,000,000	20
		150,000,000	26,000,000	17
5	GB	340,000,000	180,000,000	50
		320,000,000	206,000,000	64
6	HP	150,000,000	86,000,000	57
		240,000,000	92,000,000	38

The supernatant liquid was then decanted and the sediment re-suspended in 2 ml. of sterile skim-milk. This suspension was then frozen and dried. A routine skim-milk culture was then frozen and dried simultaneously. Viable counts were made as in previous experiments. Results are recorded in Table 6.

No marked improvement in survival rates was obtained by centrifuging the bacterial cells first and then suspending in sterile skim-milk. In the trials performed the uncentrifuged cultures had higher survival rates.

(6) Effect of Storage on Survival Populations.

Four cultures—"GB", "HP", "Q4", and "R7"—were pre-frozen and dried using the Gray technique.

Viable counts were made prior to freezing and drying and after one and two months' storage respectively. Percentage survivals are included in Table 7.

After two months the survival percentage was less than 1 per cent.

Table 6.

COMPARISON OF SURVIVAL RATES OF CENTRIFUGED AND UNCENTRIFUGED CULTURES.

Trial.	Culture.	Viable Count per 0·1 ml.		Percentage Survival.
		Before Freeze-drying.	After Freeze-drying.	
1	GB	240,000,000 230,000,000	188,000,000 173,000,000	77
	GB (Centrifuged)	370,000,000 400,000,000	227,000,000 226,000,000	59
2	HP	120,000,000 140,000,000	51,000,000 60,000,000	43
	HP (Centrifuged)	100,000,000 30,000,000	12,000,000 1,000,000	12

Table 7.

PERCENTAGE SURVIVAL AFTER STORAGE.

Culture.	Percentage Survival.		
	Before Storage.	1 month's Storage.	2 months' Storage.
GB	36	2	< 1
HP	11	1	< 1
Q4	19	1	< 1
R1	20	3	< 1

(7) Conclusions.

(*a*) Gray's apparatus is simple and easy to handle.

(*b*) Self-frozen cultures did not give survival populations as high as externally frozen cultures.

(*c*) The mortality in all drying procedures appears to be high on storage.

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(Received for publication June 19, 1959.)