Membrane Filtration: Survival of Brewing Microbes on the Membrane During Storage at Reduced Humidities[1]

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ABSTRACT

The membrane filtration technique as applied in multiplant brewery quality control has been unsatisfactory because of dehydration and subsequent death of entrapped microbes during shipment (storage) of membranes. Gram-negative bacteria and lager yeast were shown to be very susceptible to such storage over five days at humidities ranging from 0 to 98%. Gram-positive brewing bacteria, however, were much more resistant over the whole range of humidity. A number of compounds were used as protective agents in an attempt to prevent dehydration and subsequent death. One of them, 4% reconstituted skim milk powder, was extremely effective in reduction of death and is now recommended as a protective washing solution for microbes collected on membranes. Use of this protective agent allows membranes to be shipped by mail in sterile Whirl-pak bags under a variety of conditions with no appreciable microbial die-off.

Key words: *Bacteria, Dehydration, Membrane filtration, Multiplant quality control, Protection, Skim milk, Survival, Yeast*

The use of membrane filters, widespread in microbiology since World War II, has been applied to brewing since the early 1950s (8). The procedure permits a direct bacterial or yeast enumeration from large volumes of water, wort, or beer that might contain small numbers of organisms. It also provides for separation of nutrients from microbes, permitting removal of inhibitory factors, and the use of any general purpose, selective, or differential medium. In 1956 Haas (8) outlined important points for using membranes in microbiology, and membranes have since been exhaustively studied because of their importance in routine quality control both in the beverage industry (3,9,13,16,17,19,21) and in water microbiology (6,23). New developments using membrane filters include a continuous sampling method for beer (18) and continuous monitoring of wine (20).

In 1957, Clark et al (6) described a delayed incubation membrane filter test in which a local water plant filtered a sample and shipped the membrane on a preservative medium ("benzoated Endo-type") in a tin to a central laboratory for completion by incubation on Endo agar. This type of multiplant quality control monitoring in a central laboratory was also described for a brewing operation by Kovecses et al (13). These authors described a number of problems involved in shipping samples on membranes to a head office, including samples packed in the absence of nutrients, survival of entrapped organisms under adverse conditions, humidity control on subsequent incubation, and control of colony spreading (motility).

Since 1972, experience in the use of membranes for multiplant quality control has increased and techniques have been improved. Following an extensive comparison of shipped samples with those plated on site and because of the deterioration of the Canadian postal service, certain key problem areas needed reexamination. In particular, this paper will consider death resulting from dehydration of brewing bacteria on membranes and methods to protect such microbes from the effects of drying and lowered water activity (5), which are known to affect cell viability (10,18,25).

EXPERIMENTAL

Relative Humidity

Two-quart mason jars or desiccators were used as humidity chambers and were poised at stated humidity levels by the use of a large excess of various crystalline reagent grade salts (KNO_3, Li_2SO_4, NaCl, $Ca(NO_3)_2$, $KC_2H_3O_2$) in oversaturated solutions as described by Labuza et al (14) and Anonymous (1). Humidity vessels poised over 80% were carefully monitored for microbial growth and were autoclaved periodically. Membranes on which pure cultures of brewing microbes were entrapped were put into sterile Whirl-pak bags, which were left open. These were placed into the chambers or into "near 0%" humidity, which was obtained by using a jar without water but with $CaSO_4$ added. Close to 100% humidity was obtained by placing membranes directly onto 1.0% agar (no nutrients) in small plastic petri plates placed in a mason jar containing 10 ml of H_2O.

All humidities were monitored using a model L15-3050 Hygrodynamics hygrometer with the appropriate class A narrow range sensor of type TH-3, suitable for measurement of both humidity and temperature (all made by American Instrument Co., Silver Spring, MD). All incubation was at 27°C. Humidities were stabilized at least 48 hr before experimentation and were checked 1 hr before and 2 hr after the jar was opened as well as every 24 hr during experiments. All humidities were temperature corrected, using humidity calibration charts supplied with the equipment.

Organisms

The organisms used in this study, named as they were received, included representatives from those genera of concern to brewing microbiologists (12). They were *Pediococcus* sp., BSO 77 (4);

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Flavobacterium proteus, B-125 (11), now *Hafnia protea; Lactobacillus brevis*, BSO 31 (4); *Acetobacter* sp., BSO 7 (4); *Escherichia coli*, ATCC 25922; and *Saccharomyces carlsbergensis*, now *S. uvarum*, Molson Breweries of Canada Ltd., culture collection L-5.

All organisms were checked for purity by streaking for isolated colonies on tomato juice agar (Difco) plates.

To prepare suspensions of these organisms for filtration, cultures were inoculated into tomato juice broth in sidearm 250-ml Erlenmyer flasks and monitored for turbidity using a Klett Summerson colorimeter (with a No. 66 red filter, Klett Mfg., Co. Ltd., New York, NY). Using the relationship determined in this laboratory (70 Klett units $\approx 5 \times 10^8$ bacteria per milliliter or 100 Klett units $\approx 1 \times 10^7$ yeast per milliliter), cultures were serially diluted in 0.1% peptone water (22) so that filtered samples would contain approximately 150 colony-forming units if all remained viable. Replicas of this suspension were filtered using 0.45 μ 47-mm Millipore gridded S-pak membranes and rinsed with 5 ml of peptone water or wash solution as specified.

Chemicals

Sucrose and all salts used for humidity control were reagent grade, purchased from Fisher Scientific Co. (Edmonton, Alta.). Bacto gelatin and Bacto yeast nitrogen base (YNB) were purchased from Difco, (Detroit, MI). Sodium polypectate (sodium polygalacturonic acid grade II), xanthan grade II, and sodium carboxymethyl cellulose were purchased from Sigma (St. Louis, MO). Polyethylene glycol 4000 was from J. T. Baker, and the skim milk powder (spray-dried) was purchased from Dairy Producers Co-operative, Regina, Sask.

Tests

Preliminary experiments had showed that membrane-entrapped cells of genera of Gram-negative bacteria were extremely sensitive to lowered humidities. Moreover, Gram-negative bacteria have been reported to be more sensitive to reduced water activity than are Gram-positive microbes (24,25), although *Flavobacterium* sp. isolated from air may not be as sensitive to changes in humidity (7). To confirm this, an experiment was conducted with pure cultures of verified Gram-negative and Gram-positive brewing isolates. Quadruplicate samples were plated immediately onto tomato juice agar and "aged" at near 100% humidity, near 0% humidity, or at salt-stabilized humidities near 22, 45, 56, 76, 93, and 98% humidity. All membranes were aseptically transferred to the surfaces of freshly prepared tomato juice agar for enumeration after five days of storage.

For *Pediococcus* and *Lactobacillus* spp., subsequent incubation was always done in a National Appliance Co. anaerobic incubator after two cycles of evacuation and refilling with beverage grade CO_2.

To demonstrate that storage of microbes in the absence of nutrients is possible for periods approximating average postal delivery times of up to five days, a series of experiments was conducted in which 70–260 organisms of each genus were entrapped on each of eight membranes. Four were immediately placed on tomato juice agar medium and the other four were "aged" under what was considered to be as close to 100% humidity as possible; each membrane was placed on the surface of a freshly prepared 1% agar plate (with no added nutrients), where it remained for five days.

Work on viability of bacteria in dilution fluids (22) and on resuspension solutions (2,15) used in lyophilization to prevent death of freeze-dried cultures (2,15) has suggested that dehydration of organisms on stored membrane filters might be prevented by the use of "protective" agents. An initial survey was therefore conducted at 43% humidity, where $<10\%$ viability of *Acetobacter* was expected, using, where possible, two concentrations of several possible protective agents. Cells of *Acetobacter* were collected on the membrane, rinsed with 5 ml of the appropriate agent, and stored over $Zn(NO_3)_2$ at 27° C.

Protective agents showing some promise were then tested at different concentrations at 43% humidity and then over the complete range of humidities with each organism. Protective agents were also tested at extreme temperatures.

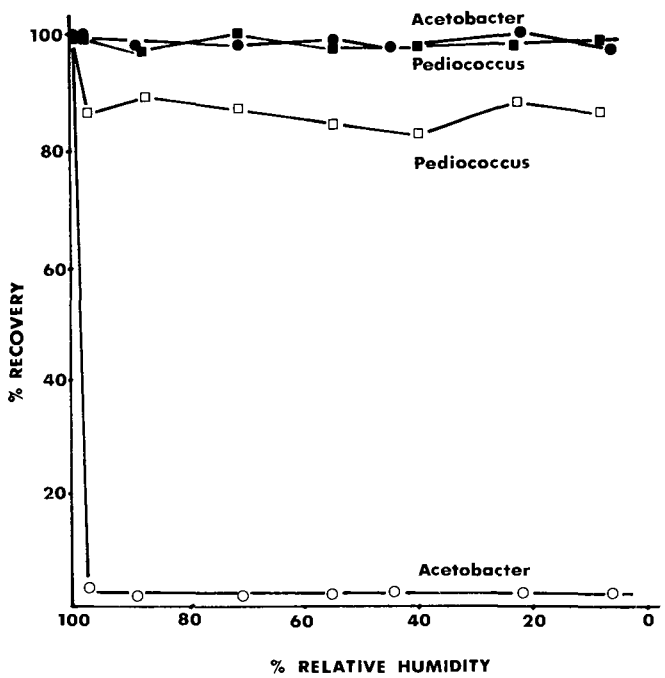

Fig. 1. Susceptibility of *Pediococcus* sp. BSO 77 and *Acetobacter* sp. BSO 7 entrapped on membrane filters to dehydration over a range of humidities. o, □ = without protective rinse; •, ■ = with protective rinse.

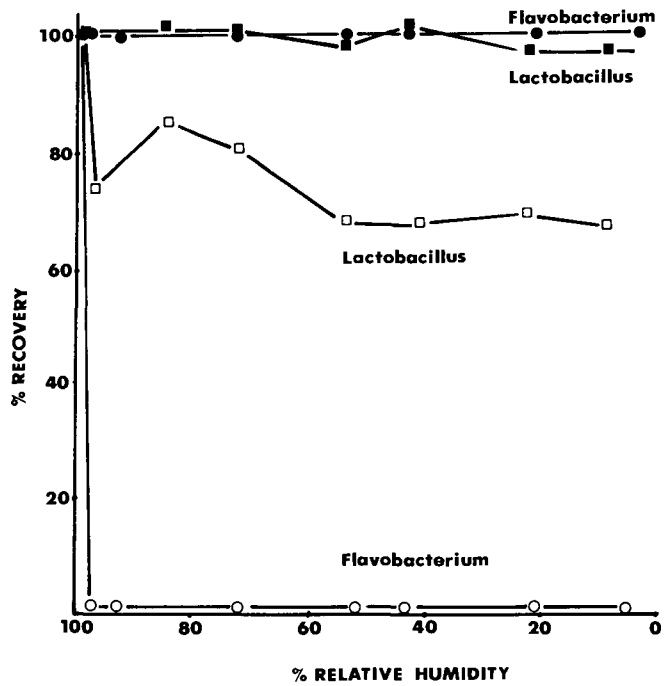

Fig. 2. Susceptibility of *Lactobacillus brevis* BSO 31 and *Flavobacterium proteus* B-125 entrapped on membrane filters to dehydration over a range of humidities. o, □ = without protective rinse; •, ■ = with protective rinse.

RESULTS AND DISCUSSION

Figures 1–3 (open symbols) show that over 70% of the Gram-positive *Pedicoccus* and *Lactobacillus* cells survived in all tested humidities. This substantiates Webb's finding that *Staphylococcus albus* and *S. aureus* (both Gram-positive) retained their viability in aerosols under a wide range of relative humidities (25). Gram-negative brewing bacteria and *Saccharomyces carlsbergensis*, however, were extensively affected at all humidities less than 100%. This loss in viability was complete for the Gram-negative bacteria, whereas 30–40% of the *Saccharomyces* survived.

These observations were not a surprise; the membrane technique as used in multiplant quality control had failed more than once to demonstrate the presence of Gram-negative bacteria previously reported in treated and untreated water supplies by normal in-plant brewery surveys.[2]

Table I shows that when organisms were stored for five days at 100% humidity without nutrients, virtually all of the genera of significance to brewing were quantitatively recovered. Therefore lowered humidity, rather than lack of nutrients, is the probable cause of death of entrapped organisms. Membranes could be shipped in 100% humidity, but that would be expensive and also unsatisfactory under freezing conditions during Canadian winters.

Table I also shows that the membrane filtration method is extremely accurate and reproducible when homogeneity of the liquid is not a problem. Each experiment was repeated twice, and the same degree of accuracy between plates in a single test was also found throughout other reported experiments.

An initial survey of possible protective agents was conducted at 43% humidity, in which *Acetobacter*, a Gram-negative rod, was expected to show little survival. Table II shows that many of the solutions looked promising, as evidenced by a higher percent recovery of organisms in higher concentrations than in lower ones. For further testing, we decided to concentrate research efforts on skim milk powder and on 20% sucrose with 0.5% YNB, a substance previously suggested as protective by Molzahn and Portno (18) without supportive evidence.

[2] Unpublished data.

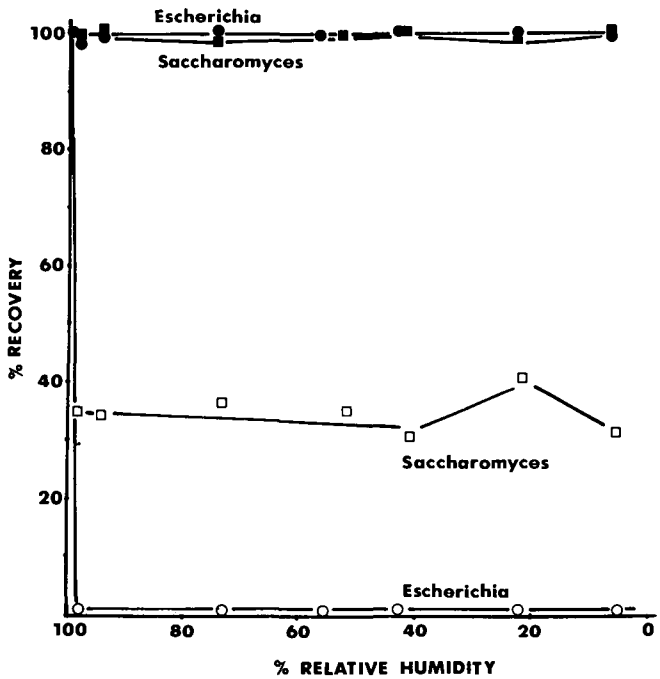

Fig. 3. Susceptibility of *Saccharomyces carlsbergensis* and *Escherichia coli* ATCC 25922 entrapped on membrane filters to dehydration over a range of humidities. o, □ = without protective rinse; •, ■ = with protective rinse.

Table III illustrates results for three of the brewing microbes previously shown (Figs. 1–3) to suffer from dehydration. This experiment was also conducted at 43% humidity using $Zn(NO_3)_2$. Under these conditions, none of the three organisms were expected to survive without protection. Table III conclusively shows that for all three microbes, 4% skim milk is an excellent protective agent; for *Flavobacterium* it is clearly a better protective compound than sucrose with YNB, which was completely ineffective in preventing death of entrapped *Flavobacterium*.

To judge the effectiveness of 4% skim milk powder over the complete range of humidities, experiments were conducted on organisms entrapped on membranes in the presence of the milk. These were conducted for all four susceptible microbes and on two more dehydration-resistant Gram-positive genera. Results are shown in Figs. 1–3 (closed symbols).

TABLE I
Survival of Typical Brewery Microbes on Membranes Stored at 100% Humidity

	Total Count[a]				
	Control		After Storage[b]		
Organism	Mean	Standard Deviation	Mean	Standard Deviation	Recovery (%)
Lactobacillus	172	5.2	161	5.0	94
	207	3.8	202	4.8	98
Pediococcus	128	2.9	130	2.1	101
	155	1.3	151	2.4	97
Saccharomyces	263	11.9	256	3.8	97
	237	4.7	227	6.1	96
Acetobacter	241	1.5	242	3.1	100
	289	2.9	293	5.1	101
Flavobacterium	195	2.5	193	2.6	99
	163	5.0	190	8.7	104
Escherichia	159	1.0	157	5.5	98
	84	4.5	81	6.5	96

[a] Colony-forming units on triplicate membranes, two separate experiments.
[b] On 1% agar with no nutrients, five days at 27° C.

TABLE II
Initial Survey for Protective Agents[a]

Protective Fluid	Concentration (%)	Average Total Count[b]		Recovery (%)
Control	...	241	(1.4)[c]	...
Near 100% humidity	...	242	(3.1)	100
Near 0% humidity	...	0		<0.4
Gelatin	0.2	1	(1.7)	<0.4
	1.0	7	(3.1)	3.0
Peptone	0.2	16	(3.6)	6.6
	1.0	13	(5.8)	5.5
Xanthan	0.05	48	(5.8)	19.6
Polypectate	0.2	40	(3.0)	16.4
	1.0	66	(11.6)	27.2
Carboxymethyl cellulose	0.2	66	(7.6)	27.4
	1.0	44	(7.0)	18.2
Polyethylene glycol 4000	10	97	(4.6)	40.0
Skim milk powder	0.2	159	(9.0)	66.0
	1.0	194	(7.0)	80.0
Sucrose/YNB[d]	20:0.5	252	(2.6)	104

[a] Experiments done with *Acetobacter*, most in quadruplicate. Membranes were aged five days at 43% humidity at 27° C, then plated onto tomato juice agar for 48 hr at 27° C.
[b] Colony-forming units per membrane.
[c] Standard deviation from the mean.
[d] Yeast nitrogen base.

TABLE III
Protection of Entrapped *Acetobacter, Flavobacterium*, and *Saccharomyces* from Storage/Shipment Dehydration at 43% Humidity on Membrane Filters

Protective Solution	Concentration (%)	*Acetobacter* Average Count[a]	*Acetobacter* Percent Protection	*Flavobacterium* Average Count[a]	*Flavobacterium* Percent Protection	*Saccharomyces* Average Count[a]	*Saccharomyces* Percent Protection
Control[b]	...	157	...	276	...	222	...
100% humidity	...	156	100	274	99	224	101
Near 0% humidity	...	1	0.8	0	<0.3	9	4
Sucrose with 0.5%	0[c]	46	29	164	59	146	66
Yeast nitrogen base	2	110	71	0	<0.3	135	61
	5	118	75	0	<0.3	152	68
	10	120	77	0	<0.3	154	69
	15	127	81	0	<0.3	222	100
	20	158	101	0	<0.3	219	99
Skim milk	0[c]	6	4	0	<0.3	23	10
	0.2	11	7	18	7	54	24
	0.6	36	23	38	14	58	26
	1.0	100	64	93	34	100	45
	2.0	118	76	276	100	224	101
	3.0	146*	94	276	100	222	100
	4.0	ND[d]	...	278	101	222*	100

[a] Colony-forming units per membrane (standard deviation <7.0 except where marked *).
[b] H_2O or peptone wash.
[c] Recovery control at 43% humidity.
[d] Not determined at this time, 100% protection in later experiment.

Figures 1 and 2 clearly show that Gram-positive *Pediococcus* and *Lactobacillus* are fully protected by a 4% skim milk rinse, which ensures quantitative recovery over the complete range of humidity. More dramatically, when skim milk was used as a protective agent, the Gram-negative rods and *Saccharomyces* that had previously been killed by dehydration were virtually 100% viable regardless of the humidity. This is solid evidence to suggest that a protective wash is mandatory before membrane shipment in a multiplant quality control program.

In addition to the experiments at 27° C, more rigorous temperature extremes of 35 and −24° C and a series of freeze-thaw cycles were used for membrane storage of *S. carlsbergensis* and *F. proteus*. Although humidities changed drastically when jars were altered in temperature to such extremes, in the lowest possible humidty (near 0%), the use of skim milk wash enhanced recovery at −24° C from 43 to 76% (*Flavobacterium*) and from 1.5 to 18.7% (*Saccharomyces*). Under freeze-thaw conditions, most of the *Flavobacteria* were killed, and at near 100% humidities, recovery ranged from << 0.5% (water wash) to 1.9% (skim milk). *Saccharomyces* lager yeast was less affected; skim milk increased recovery to 31 from 8%. Data from the salt solutions is not reported because of difficulties in assessing actual humidities under these extremes of temperature. Interestingly, however, neither organism survived under any conditons at 35° C! Membranes shipped by mail for multiplant quality control should therefore be protected as much as possible from environmental extremes; room temperature shipment appears best.

Luckily, 5–7 ml of skim milk at 4% w/v pass through a 0.45-μ membrane with no difficulty, and this is sufficient to rinse filters used for samples of 10 ml or less. Much variation in flow rate appears to exist between lot numbers of membranes.[3] Some lots can be washed by much larger volumes of skim milk rinse. Larger beer or water samples that wet the membrane holder would require a prerinse with sterile water or 0.1% peptone to ensure 100% collection of sample microbes on the membrane. This can be followed by the skim milk rinse. Skim milk can be easily reconstituted and autoclaved. Its only drawbacks as a membrane washing solution are that more than 7–10 ml will clog some 0.45-μ membranes and that it will support the growth of microbes if not sterile or not handled aseptically.

In summary, we recommend that all membranes used to collect microbes that will not be plated for some time (as in the described multiplant quality control program) should be rinsed immediately with 5–7 ml of 4% skim milk to protect entrapped microbes from dehydration and subsequent death. Humidity control during storage and shipment is probably far more important than is the control of humidity on incubation (13). Incubators with water reservoirs easily maintain humidities from 50–98% (depending on type) in the macroenvironment; the microenvironment in the petri dishes is near 100%. On the contrary, the microenvironment of the membranes shipped in individual sterile Whirl-pak bags approximates 7%. The effect of this dehydration can be circumvented by the skim milk protection described here.

[3] More uniform flow is provided by HAMK membranes.

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